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Studies during the current granting period lead to the following major findings: (1) identification of a novel tumor Ag for breast and ovarian CTL from the folate binding protein (FBP) with a dominant HLA-A2 epitope E39 (FBP, 191-199); (2) identification of Th1 epitope from HER-2 that induces IFN-γ preferentially when recognized in association with HLA-DR4; (3) establishment of approaches for (a) culture of breast TIL from small primary tumors and (b) HPLC fractionation and epitope reconstitution from breast tumors.

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PROGRESS REPORT:

Introduction

Development of biological therapies for cancer in recent years has generated new hopes that improved cancer cure rates can be achieved beyond what is currently obtained with combinations of chemotherapy and radiation therapy. Biological therapies use tumor Ag and cytokines (or their genes) with the objectives: **(1)** to induce tumor specific CTL and augment Ag presentation to anti-tumor effectors, **(2)** to ensure full activation of anti-tumor immunity and **(3)** to achieve optimal proliferation and expansion of specific anti-tumor effector T cells (1).

Since CTL epitopes from tumor antigens are short peptides generated from self-proteins (2), to address the first objective, ongoing studies needed to accomplish two major tasks: **(a)** to characterize the tumor Ag either by mapping with synthetic peptides or by sequencing of the naturally processed peptides presented by the tumor (1, 2), and **(b)** to optimize the epitope sequence for binding to the Ag presenting molecules (which are the human histocompatibility antigens A, B, C - HLA, A, B, C), and for enhanced recognition by the corresponding receptor (the T cell antigen receptor, TCR) of the effector cytotoxic T lymphocyte (CTL) (3).

The need for these studies is dictated by a number of factors which are: **(a)** transfer of viable/irradiated tumor cells in a human organism raises serious concerns regarding transfer of genetic material to the recipient. This genetic material (DNA, RNA) contains oncogenes which may induce metaplastic or neoplastic transformation of healthy tissues. Thus peptides, or genes encoding the peptides of interest substituting for the tumor may provide new approaches to immuno- or gene therapy that circumvent this problem. **(b)** therapy with cytokines alone or with live cells transduced with cytokine genes has not been shown to induce systemic anti-tumor immunity (reviewed in 4, 5). Of additional concern is the possibility that systemic administration of cytokines alone may activate primarily auto-immune reactions and processes which may not be directed to the tumor. Effective activation of naive CD8+ cells requires co-stimulation e.g. through the B7-CD28 pathway (6-8). The co-stimulatory

molecules B7.1/B7.2 are up-regulated by cytokines (9, 10). Thus, focused cytokine secretion after specific and well defined cellular interactions may provide a more suitable approach to control the activation of anti-tumor effectors. This can be accomplished by using as "activators" the T helper cells (Th) of Th1 memory phenotype (6). These Th1 cells can recognize the tumor Ag in the context of major histocompatibility Ag class II (i.e. HLA-DR, HLA-DQ) and secrete a specific pattern of cytokines: IFN-γ, IL-3, TNF-β, which are involved in the maturation and effector function of cellular immune cytotoxic effectors such as CTL (11). **(c)** The epitopes defined on tumor Ag are self-peptides of significantly lower binding affinity for both HLA and TCR of the responding CTL. Thus, their efficiency for tumor specific CTL induction will be low.

As a results of our studies conducted under this grant (12), the immunodominant HER-2 peptide E75 is now tested in a first phase Ib clinical trial for an equal number of breast and ovarian cancer patients. This clinical trial started in August 1997 and we anticipate that the first results will become available in the summer of 1998.

The gene for HER-2 is overexpressed in approximately 50% of breast cancer patients. This raises the need for identification of additional epitopes from other Ag that can target CTL effectors to HER-2^{lo} tumors or tumor variants that arose from immunoselection mechanisms, i.e. $E75^{-}$ tumor cells can escape the attack of E75 specific CTL, and are at a proliferative advantage.

The objectives of our studies during this grant period were four fold: **(1)** To establish whether tumor peptides can be isolated, HPLC fractionated and recognized by CTL associated with breast tumors. These studies continue the direction established in our previous report, where the line SKBR3.A2 was successfully transfected and selected with the antibiotic G418 to express HLA-A2; **(2)** To define conditions for long-term propagation and expansion of CTL isolated from breast tumors; **(3)** To identify peptides from the HER-2 protein

that are endowed with T cell stimulatory activity and Th1 (T helper 1) cytokine (IFN-γ, interferon gamma) secretion. The responder T cells were of breast cancer patients with primary tumors. **(4)** To identify novel tumor antigens recognized by CTL in ovarian and breast cancer. **These studies lead to the identification of a novel tumor antigen defined by the folate binding protein (FBP); we have also identified an immunodominant epitope from FBP defined by the peptide E39, FBP (191-199). (5)** To establish whether increased drug-resistance of breast and ovarian tumor cells leads to enhanced sensitivity by tumor-specific CTL-TAL.

The results of these studies and their significance are presented in Body of the report.

Body of the Report

The research performed during the last twelve months has made significant progress towards the goals of this study and the overall goal of developing specific immunotherapy for breast cancer. The results obtained are as follows:

1. The purpose of the present work continues to be the characterization of the functional significance of the CTL epitopes as potential antigens for targeted immunotherapy. The studies during this granting period focused on the well defined tasks. The HLA-A2 transfected breast cancer line SKBR3.A2 (13) is currently used for Ag characterization. To characterize naturally processed peptides we defined the conditions for separation of active peptides. We developed a set of successive gradients of acetonitrile (ACN) (14, 15). Thus, peptides extracted from immunoaffinity purified HLA-molecules from 2 x 10^9 cells were separated through a first gradient and the position of the active peaks was identified. Separation conditions were modeled for each fraction using a shallow gradient of ACN and a much longer column (21 x 3 cm) for high pressure liquid chromatography (HPLC). Studies conducted during the previous year have shown that HPLC fractionation and separation of tumor peptides from ovarian tumors is feasible. The results of the current studies show the efficiency of our HPLC gradients in separation of CTL active peptides from the breast tumor line SKBR3.A2 **(Figure 1A, 1B)**. Four major peaks of biological activity were identified to be presented by HLA-A2 to breast CTL in the first dimension. Each peak was collected, pooled, and refractionated on the second dimension HPLC using increasing gradients of increasing concentrations of Acetonitrile (Please see **Legend to the Figure 1B**). The results show that at least 12 peaks of bioactive peptides can be separated from the SKBR3.A2 cells. These results are extremely significant for further analysis of the immunogenicity and antigenicity of breast cancer. They demonstrate that breast tumors are antigenic, and a large number of these antigens can be

isolated. Ongoing studies in collaboration with the Laboratory of Drs Victor Engelhard and Dr. Donald Hunt (University of Virginia) and Argonex Corporation will attempt to elucidate the sequences of these peptides.

(2) To address the questions raised under the second task of these studies, we isolated, expanded, and characterized breast reactive CTL lines and clones. In the present studies, we have also isolated tumor infiltrating lymphocytes (TIL) and tumor associated lymphocytes (TAL) from solid tumors and malignant effusions respectively, of 29 breast cancer patients (16). Significant in vitro proliferation and expansion was observed in 22 of 29 distinct samples. The TIL cultures were initiated using OKT3 mAb in the presence of moderate concentration (25-50 U/ml) of IL-2 followed by 100 U/ml of TNF-α. TAL were not stimulated with OKT3 mAb. Six of eight distinct TAL grew in culture as predominately $CD4^+$ lines. In contrast, due to the small sample size, only 14 of 21 (66%) of primary breast TIL expanded in culture. They were predominantly of $CD8^+$ phenotype. Autologous tumor lysis was observed in seven of eight cases tested. Three of the eight TIL/TAL preferentially lysed autologous tumor. Four breast CTL lines recognized by the breast tumor line SKBR3.A2 suggesting that they may recognize peptides presented by HLA-A2. HER-2 peptides E75, C85, E89 GP2, and E90 were recognized, suggesting that multiple HER-2 epitopes are presented to breast TIL/TAL in the tumor environment. This may be of interest in developing vaccine strategies for therapeutic management of breast cancer.

The results of our studies show for the first time a reproductible approach for expansion of breast TIL and TAL, with minimal intervention in terms of enzyme digestion, restimulation with autologous tumor/OKT3 mAb, and addition of exogenous cytokines. The non-enzymatic approach is complementary to the enzymatic digestion. In fact, three breast TIL (TIL-12, -13 and -14) were isolated from samples subjected in parallel to enzymatic and non-enzymatic processing, of which only the latter was successful. Of the established TIL cultures from primary tumors, 9 were propagated from TIL

isolated by enzymatic digestion, while five were propagated without enzymatic digestion. TIL from five additional patients isolated by enzymatic digestion failed to grow in culture. We have observed significant T cell proliferation in 22 of 29 cases attempted (76%) as well as in TIL from small tumors in which restimulation with autologous tumor cells could not be performed. Our results show that breast CTL-TIL can be propagated with high efficiency even from small tumor samples after the original stimulation with autologous tumor and OKT3 mAb and expanded into large numbers.

The samples were collected at random and cultured without knowing the HLA-type of the donor. Overall, 9 of the 14 growing TIL were HLA-A2 (64%). Although the sample size is small, this suggests a proliferative advantage for T cells infiltrating primary breast tumors in HLA-A2+ patients. In TAL, HLA-$A2^+$ cultures were four of eight (50%). The phenotype of cultured TIL showed a different picture from the phenotype of TAL. 9/14 primary TIL expanded as predominately $CD8^+$ lines (with $\geq$ 50% $CD8^+$ cells in the population) while 1/8 TAL were predominately $CD8^+$. The different outcome in the phenotypes may suggest an active priming and stimulation *in situ* of $CD8^+$ in primary tumors. The percentage of $CD8^+$ cells was higher in cultured TIL isolated without enzymatic digestion (4/5, 80%) than in samples propagated after enzymatic digestion (5/9, 56%). Since the average of $CD8^+$ in the peripheral blood ranges between 25 - 33%, this suggests an active recruitment of $CD8^+$ cells at the primary tumor site (17), compared with the pleural effusion/ascitic TAL, where contamination from passenger lymphocytes may be higher. This approach may be also useful to identify the dominant peptide specificities of tumor associated CTL. The HER-2 peptides recognized may reflect Ag that have been stimulatory during tumor progression, but either not sufficiently strong to elicit a curative response, or novel tumor variants were selected. These variants may not express these Ag in sufficient amount to sensitize CTL for killing, or may express other Ag. It should be mentioned that all tumor Ag reported to date are recognized with low affinity (18).

Identification of peptides recognized among the pool of candidate epitopes may allow development of vaccines to amplify a CTL response to breast cancer earlier, when the tumor is small. Conversely identification of specificities amplified after repeated stimulation of TIL with autologous tumor (when available) may be useful for adoptive therapies with CTL-TIL plus cytokines. These therapies may be more suitable for patients with advanced disease who are less likely to respond to cancer vaccination *(Please see appended manuscript).*

(3) Previous studies have characterized the reactivity of $CD8^+$ CTL with ovarian and breast cancer. There is little information about the antigens and epitopes recognized by $CD4^+$ T cells in these patients. We analyzed the ability of T cells from PBMC of breast cancer patients to recognize HER-2/neu (HER-2) peptides. We found that 13/18 patients responded by proliferation to at least one of the HER-2 peptides tested. **Of these peptides, one designated G89 (HER-2:777-789) was recognized by T cells from 10 patients. 7/9 responding patients were HLA-DR4$^+$, suggesting that this peptide is recognized preferentially in association with HLA-DR4.** Analysis of the specificity and restriction of the cytokine responses by G89-stimulated T cells revealed that these cells secreted significantly higher levels of IFN-γ than IL-4 and IL-10 suggesting priming for a Th0-T helper 1 (Th1) response. The same pattern of responses was observed to the intracellular domain (ICD) of HER-2 suggesting that G89-stimulated T cells recognized epitopes of the HER-2 protein in association with HLA-DR4. Since HLA-DR4 is present in 25% of the humans, characterization of MHC-class II restricted epitopes inducing Th1 responses may provide a basis for the development of multipotent HER-2 based cancer vaccines against breast and ovarian cancer that can activate both CTL and Th1 cells *(Please see appended manuscript).*

(4) We have investigated for CTL recognition peptides derived from folate binding protein (FBP) which is overexpressed in 90% of ovarian cancer, in

breast cancer and the majority of epithelial tumors. The significance of FBP for cancer therapy rests on the fact that FBP (also known as trophoblast antigen) appears to be overexpressed in response to folic acid/folate acid in tumor analogs. A number of candidate peptide was selected based on the concordance of HLA-A2 binding motifs and of the amphiphilic sites. The sequence of these peptides is as follows:

Code	Position	Sequence								
E37:	25-33	R	I	A	W	A	R	T	E	L
E38:	112-120	N	L	G	P	W	I	Q	Q	V
E39:	191-199	E	I	W	T	H	S	T	K	V
E40:	247-255	S	L	A	L	M	L	L	W	L
E41:	245-253	L	L	S	L	A	L	M	L	L

To obtain a better picture on the recognition and specificity of the FBP peptides cultured ovarian (4 patients) and breast (2 patients) TIL were tested within one week after isolation. Our results show that peptide E39 was recognized significantly by 6/6 CTL-TAL cultures suggesting that it is immunodominant. The peptide E41 was recognized by 3/6 TAL suggesting that is subdominant. The specificity of this response is illustrated by the fact that peptide E40 which differs from E41 only in the two N-terminal residues was not dependent on the MHC-I binding affinity. Peptide E38 of the highest binding affinity to HLA-A2 was not recognized. *(Two recent abstracts presenting these results are appended).*

(5) Studies performed on the second task have focused on characterization of the effects of a novel multidrug resistance (MDR) reversal agent (N-myristoylated PKC-α pseudosubstrate) peptide on the reversal of MDR. PKC plays a significant role in the MDR phenotype of cancer cells. The drug transport-related event that is a component of the role of PKC in MDR is PKC-induced expression of the P-glycoprotein(PgP)-encoding gene mdr1. Last year we established that the N myristylated PKC-a pseudosubstrate peptide reverses P-gp-dependent MDR in human breast cancer cells. Clinical MDR

encomposses Pgp dependent and independent mechanisms in breast and other epithelial cancers. To determine whether PKC inhibition could reverse Pgp independent MDR in cancer cells we employed a human colon cancer model of Pgp-dependent MDR. We analyzed the effects of selective PKC activators/inhibitors on the uptake of radiolabeled cytotoxic drugs by cultured human colon cancer cells that lacked Pgp activity and did not express the drug efflux pump at the level of message (mdr1) or protein. We found that the selective PKC activator 12-0-tetradecanoylphorbol-13-acetate (TPA) significantly reduced uptake of drugs in human colon cancer cells devoid of Pgp activity, and that PKC-inhibitory N-myristoylated PKC-α pseudosubstrate synthetic peptide potently and selectively induced uptake of the cytotoxic drugs in the phorbol ester-treated and nontreated colon cancer cells. TPA treatment of the cells did not induce expression of either Pgp or its message mdr1. **These results indicate that PKC activation can significantly reduce the uptake of multiple cytotoxic drugs by cancer cells independently of Pgp, and that N-myristoylated PKC-α pseudosubstrate peptides potently and selectively induce uptake of multiple cytotoxic drugs in cultured human colon cancer cells by a novel mechanism that does not involve Pgp and may involve PKC isozyme inhibition. Thus, N-myristoylated PKC-α pseudosubstrate peptides may offer a basis for the development of agents that reverse intrinsic drug resistance in human colon cancer.** This will allow continuation of studies proposed under the second task, i.e. characterization of the effects of drug resistance on the sensitivity to lysis and recognition by tumor specific CTL. We are now selecting the SKBR3.A2 cell line with Adriamycin to generate drug resistant variants of this breast cancer line. A recent study indicated that drug resistance can induce immunological resistance for NK cells (19).

To address the question whether induction of drug-resistance enhances target susceptibility to CTL lysis, first a number of ovarian and breast tumor lines were cultured with Adriamycin and selected with 250 ng/ml ADR. The results in Table I show that for most cell lines (expecting the HLA-A, B, C^{lo}

tumor MBA 453) Adr treatment increased expression of MHC-I on the cell surface. This increase was not paralleled increase in HER-2 expression or of the adhesion molecule ILAM-1 suggesting that possibility it was specific for MHC-I. The results in **Figure 2A, B** show that lysis of Adr resistant tumors 2774 and SKOV3 was higher than of their drug sensitive counterparts. This lysis was inhibited by anti MHC-I mAb (W6/32) suggesting that the breast TAL effectors recognize targets in the context of MHC-I. These effectors share with the targets HLA-A2. Furthermore we conducted immunoselection experiments. The ADR sensitivity (in MTT assay) of 2774-Adr, 2774 (wild-type) and of the 2774-Adr cells cultured with BR-TAL is shown. 2774 cells obtained after selection with TAL show: higher sensitivity to ADR than parental 2774-ADR cells suggesting that CTL-TAL preferentially lyse drug-resistant. The most likely explanation is that treatment with ADR may be increasing the expression not only of the mdr1 but also of the related TAP1 and TAP2 genes. Increased transport of peptides in endoplasmic reticulumn may increase not only the amount of peptides delivered but also stabilizing the MHC-I expression through increased availability of peptides. Ongoing studies aim to address this question.

Conclusions

The completed research during the last three years has made, we believe, significant contributions to the field of breast cancer immunology. *First*, studies in the first year of award have identified an immunodominant epitope E75 from the HER-2 which is preferentially targeted by the tumor reactive breast CTL. The peptide reconstructing this epitope now forms the basis of a new clinical trial (phase 1b) in patients with breast and ovarian cancer. Thus, the first group of findings reported last year will be soon translated to the clinic. *Second*, a novel tumor Ag has been identified in the folate binding protein. *Third*, we have established the methodological approaches and successfully isolated and fractionated peptides from the breast tumor line SKBR3.A2. These peptides are recognized by breast associated CTL. To our knowledge this is the first demonstration of breast tumor peptides isolation and recognition by human breast associated CTL. *Fourth*, the FBP derived peptide E399 is recognized by freshly isolated TAL from ovarian and breast cancer patients suggesting in vivo expression and sensitization. Since FBP is over-expressed 20-fold in most epithelial carcinomas, E39 may be the best currently known peptide to utilize in a peptide-based vaccine for epithelial tumors. **Furthermore now a polyvalent vaccine for induction of CTL specific epithelial cancer could be formed using peptide representing dominant and subdominant CTL epitopes from several proteins (HER-2, AES, FBP) and T helper peptides.**

Fifth, we have conducted an extensive study or the feasibility of isolation and expansion in vitro of breast CTL from both primary tumors and malignant ascites. We succeeded in propagating in culture twenty-two of twenty-nine breast CTL lines from different patients. The approach we used namely, tumor disruption, with limited/or without digestion followed by brief OKT3 mAb stimulation and moderate IL-2 is routinely currently used in our laboratory. This approach avoids the use of high concentrations of cytokines, or successive stimulations with tumors. It is useful for expansion of breast CTL from small

(<1.0 m) breast tumors. These breast CTLs are used for antigen characterization.

Our ongoing studies were also focused on primary CTL induction using as a model the HER-2 peptide E75, and its mutated analog F46. We used as APC (antigen presenting cells) autologous dendritic cells which were expanded in GM-CSF and IL-4. We found that in addition to IL-2, TNF-α, and IL-12 provide significant help for primary CTL induction. The presence of co-stimulatory molecules B7.1/B7.2 it is important for CTL proliferation but not for induction of CTL activity. The CD40-CD40L does not appear to be essential for induction of CTL. With the identification of helper epitope G89 this may allow development of combination CTL epitope helper epitope for development of cancer therapies. Preliminary results in this direction show that G89 can induce E75 concentration dependent IFN-γ secretion.

Future work will focus on continuing the objectives of this study. An important aspect which arises is the ability of these peptides to induce anti-tumor responses. This will focus on the groups of breast cancer patients with no evidence of disease. This group of patients is of particular interest because after first line surgery, chemotherapy and/or radiotherapy, they have no evidence of tumor. Consequently since it is predictable that over the following years many of these patients will experience relapses, this interval offers a window of opportunity to attempt to induce protective immunity to tumor. This will better address the main problem of developing novel therapies for breast cancer by intervening earlier, and not after it may be too late (metastases).

In addition to the study of CTL induction by peptides as we described, we are currently establishing the approaches for the use of adenovirus and vaccinia based vectors for expression of tumor Ag. The recent developments in the field make these viruses important tools for the study of Ag expression and processing in non-proliferating APC (adenovirus) and proliferating APC

(retroviruses) respectively. We have established the material transfer agreements (MTA) with Genzyme Corporation (Dr. Bruce Roberts) for transfer of their proprietary recombinant viruses in our laboratory. Furthermore recombinant-vaccinia containing the E75 peptide and the E75-ER (endoplasmic reticulumn translocation sequence have been prepared for us by Dr. Jonathan Yewdell from NIAID-NIH. We have submitted the protocols for the use of these viruses to the BioSafety Committee of M.D. Anderson. We anticipate that the use of these approaches will establish the feasibility of epitope specific induction of tumor immunity to breast cancer.

Legends to the Figures

Figure 1. (A) Fractionation of naturally processed peptides from the line SKBR3.A2. First dimension HPLC, column 2.1 x 30 mm. Linear gradients of ACN, 0-60 min. (-) CTL indicated the toxicity levels of each fraction the first dimension. **(B)** Second dimension separation of the peaks 1-4 separated in the first dimension. A, B, C, D individual peaks of activity. **(C)** Peptide profile (OD220 for peptide bound) separated in the second dimension HPLC. The gradients in TFA used were as follows: Gradient I: 0-5 min, o-15% ACN, 5-45 min 15-35% ACN, 45-60 min, 35-60% ACN; Gradient II: 0-5 min 0.1% TFA in H20, 5-15 min 0-20% ACN in 0.1% TFA, 15-55 min 20-40% ACN, in 0.1% TFA, 55-60 min, 40% ACN, in 0.1% TFA; Gradient III: 0-10 min 0-30% ACN, 10-50 min 30-50% ACN 50-60 min 50-60% ACN. Gradient I was used for separation of both Peaks I and II.

Figure 2. Enhancement of lysis of the ADR-resistant tumor lines, 2774 and SKOV3 by breast TAL-BA. Effectors and targets shared HLA-A3.

Figure 3. MTT assay of 2774 cells selected with ADR, or selected with ADR followed by immunoselection with breast TAL-BA. The resulting 277-ADR/CTL show similar concentration-dependent sensitivity to Adriamycin, suggesting that immunoselection with CTL preferentially removes the Adriamycin resistant cells.

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(9) Appendices

List of Appended Materials

Papers

1. Fisk, B., DaGue, B., Seifert, W., Kudelka, A., Murray, J.L., Wharton, J.T., and **Ioannides, C.G.** Mass-spectrometric analysis of naturally processed peptides recognized by ovarian tumor associated CD8+ CTL. *International Journal of Oncology* 10:159-169, 1997.

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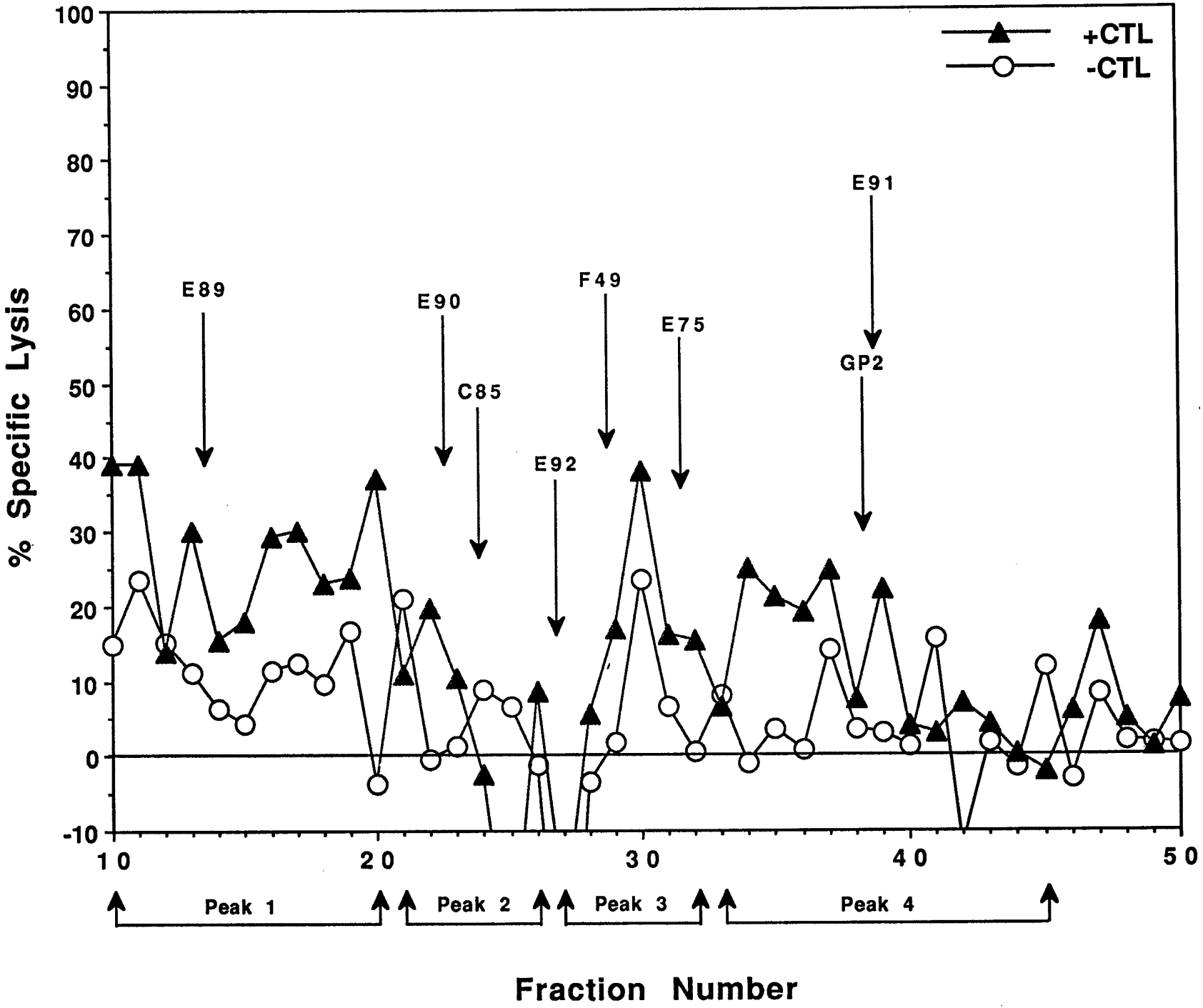
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-CTL
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100
90
80
70
60
50
40
30
20
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0
-10
E89
E90
C85
E92
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E91
GP2
10
20
30
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50
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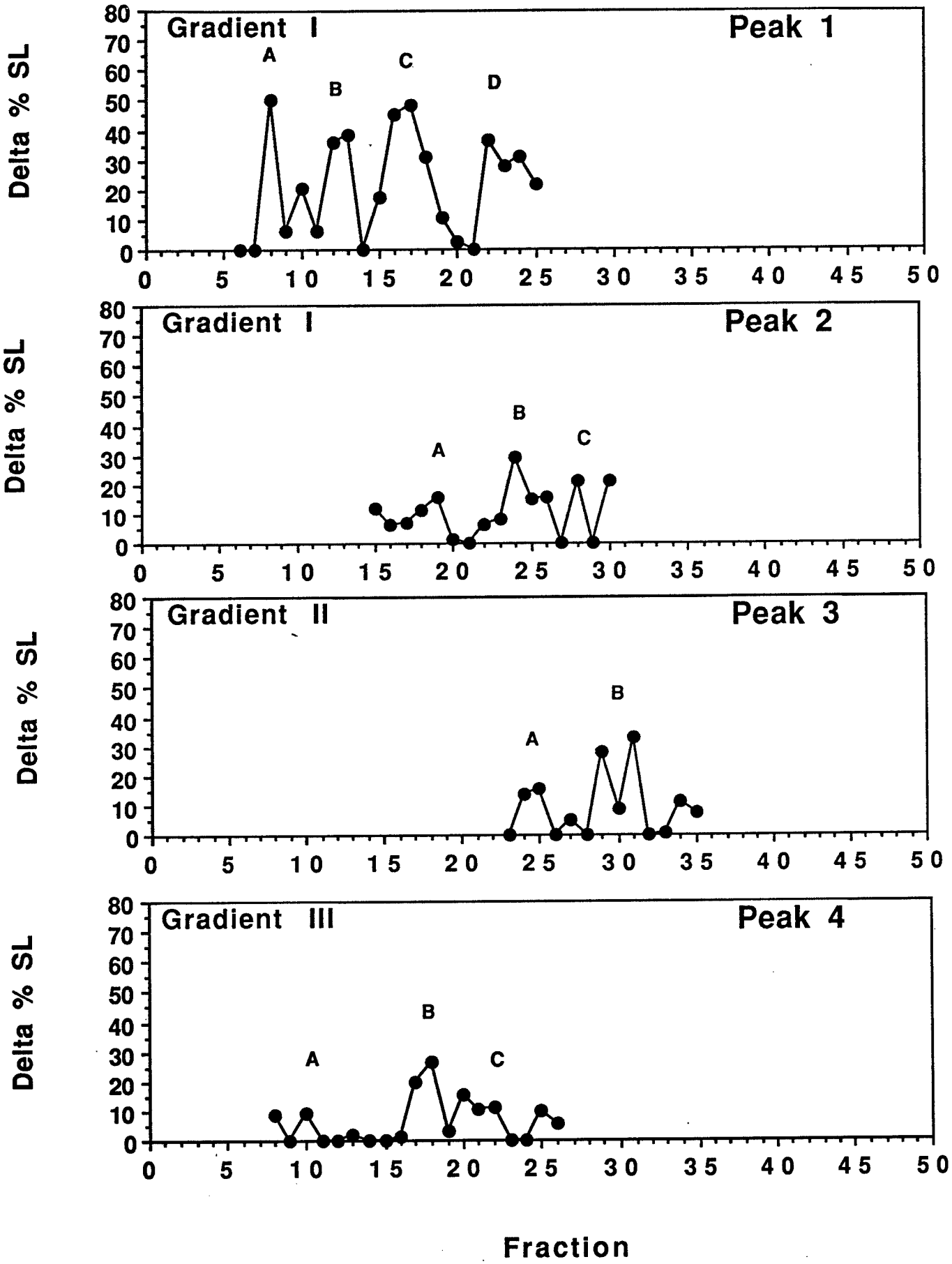
Peak 3
Peak 4
Fraction Number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Delta % SL
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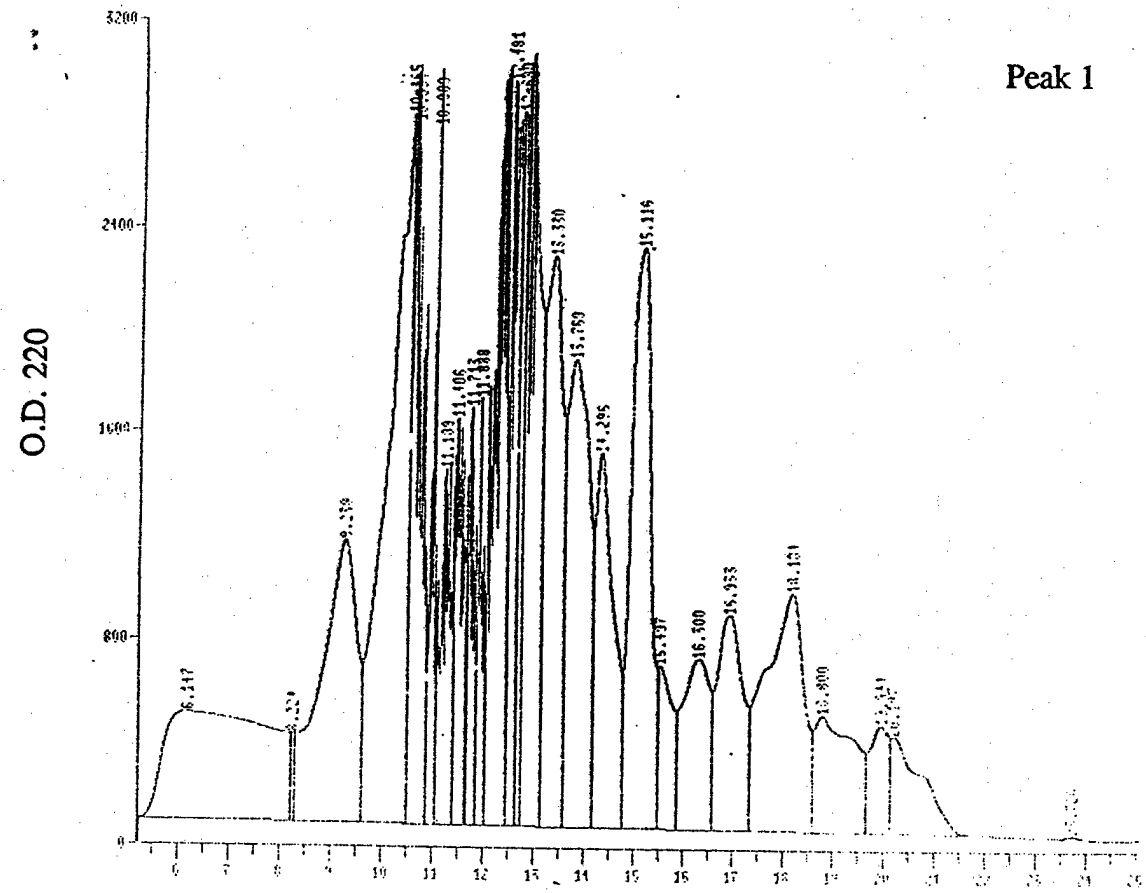
Fraction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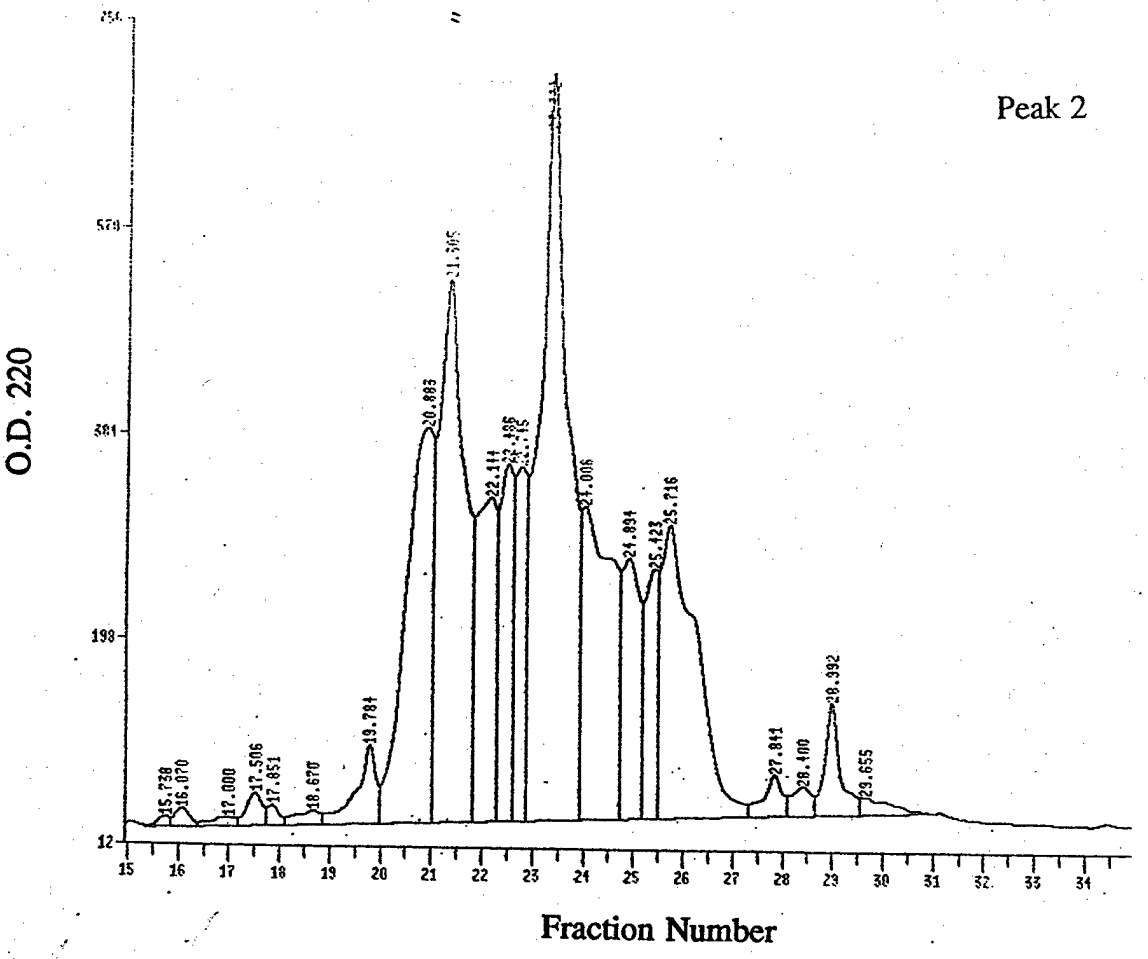
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O.D. 220
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198
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17.851
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21.395
22.111
22.486
23.015
24.006
24.834
25.423
25.716
27.844
28.100
28.392
29.655
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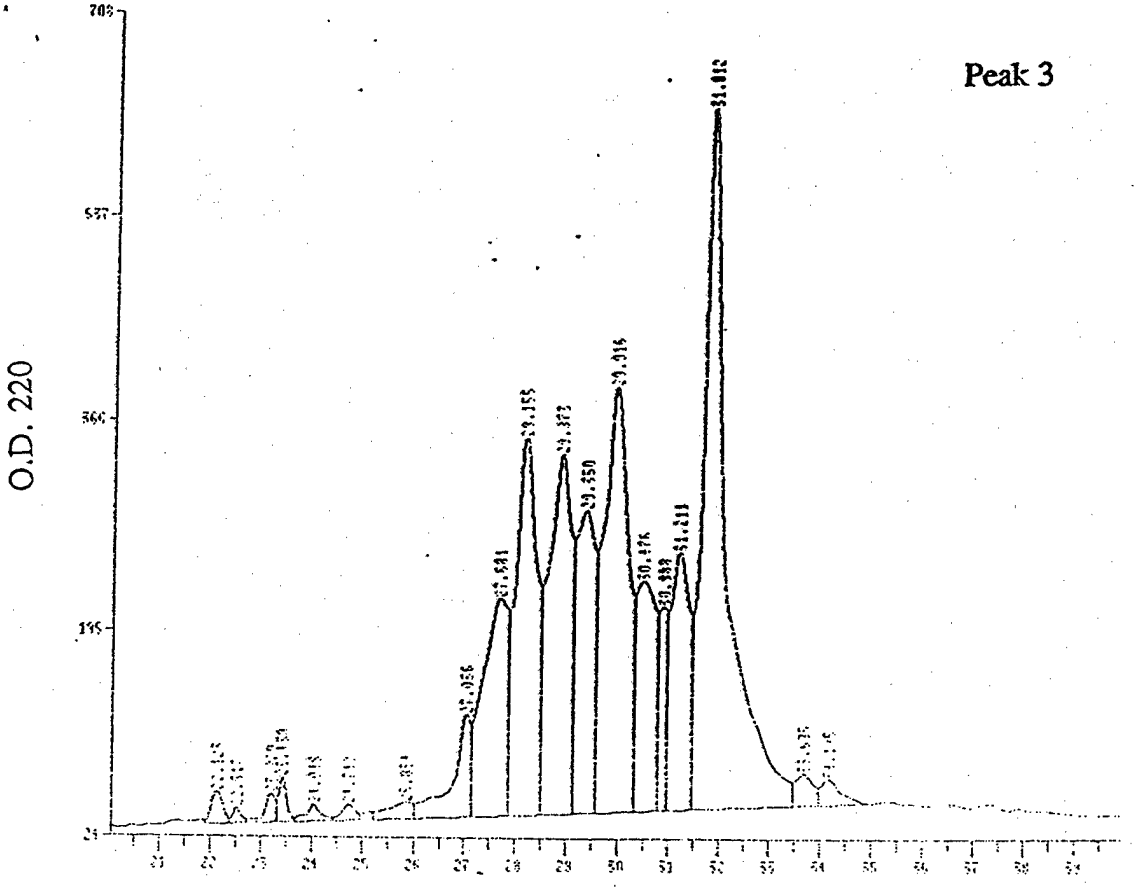
Fraction Number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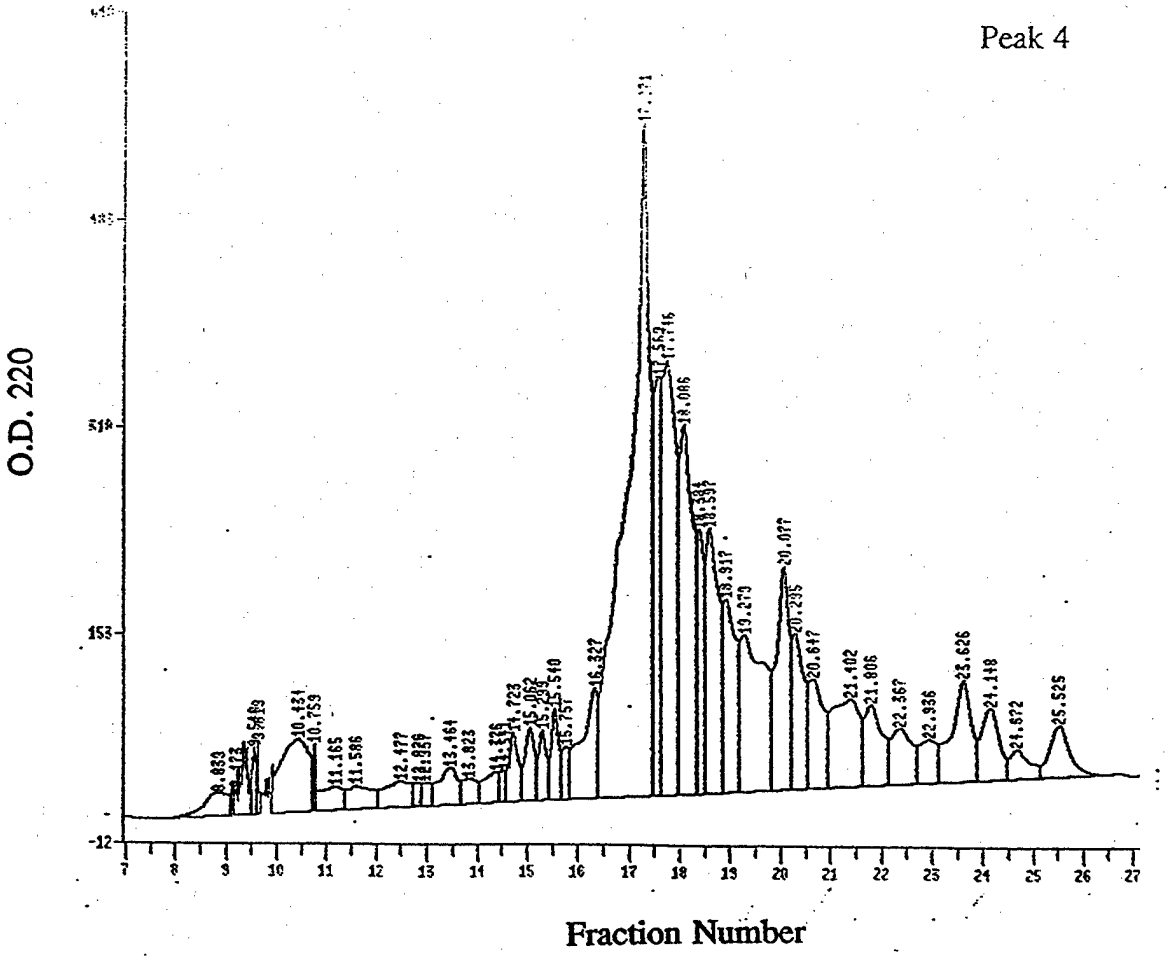
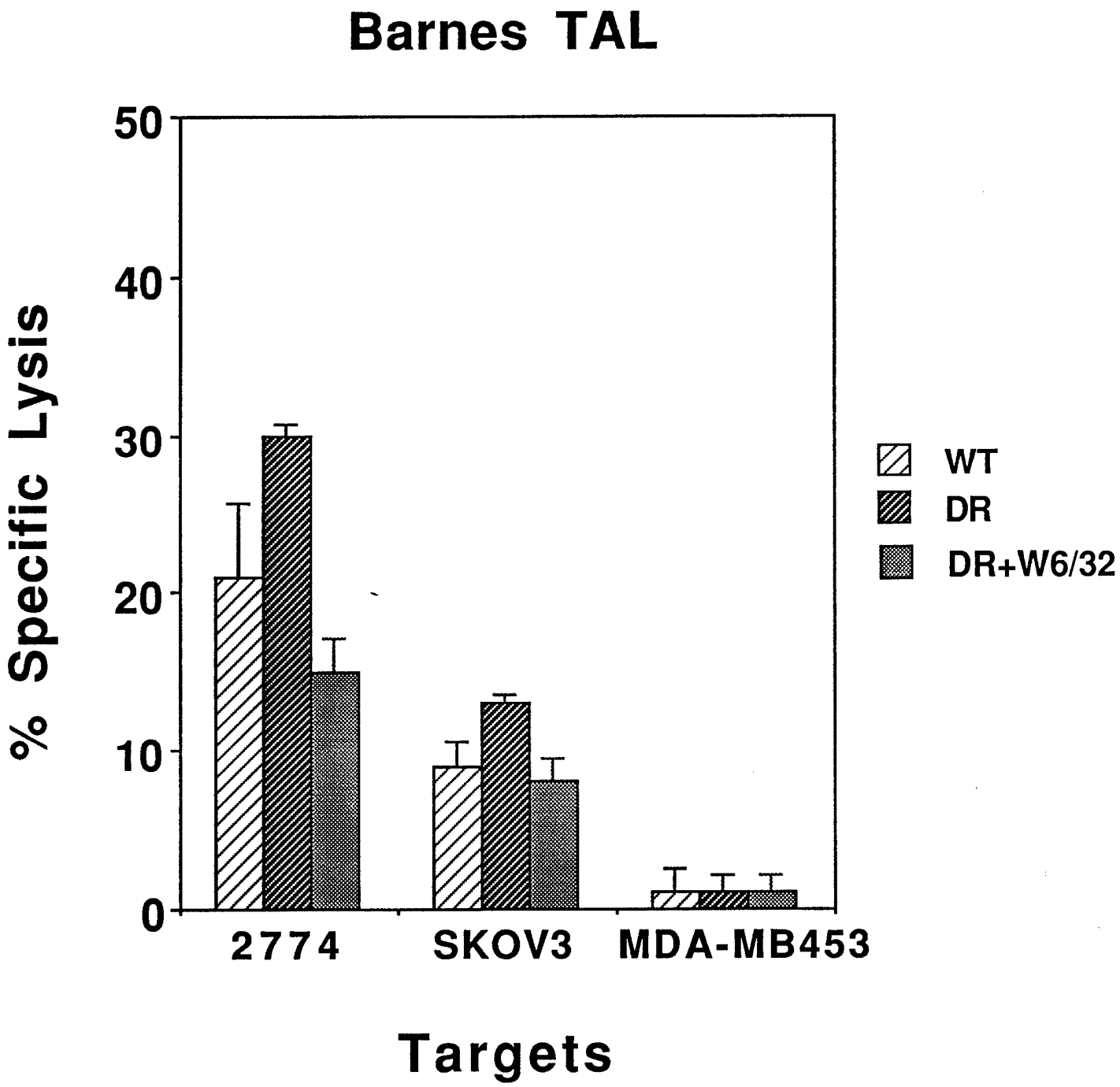
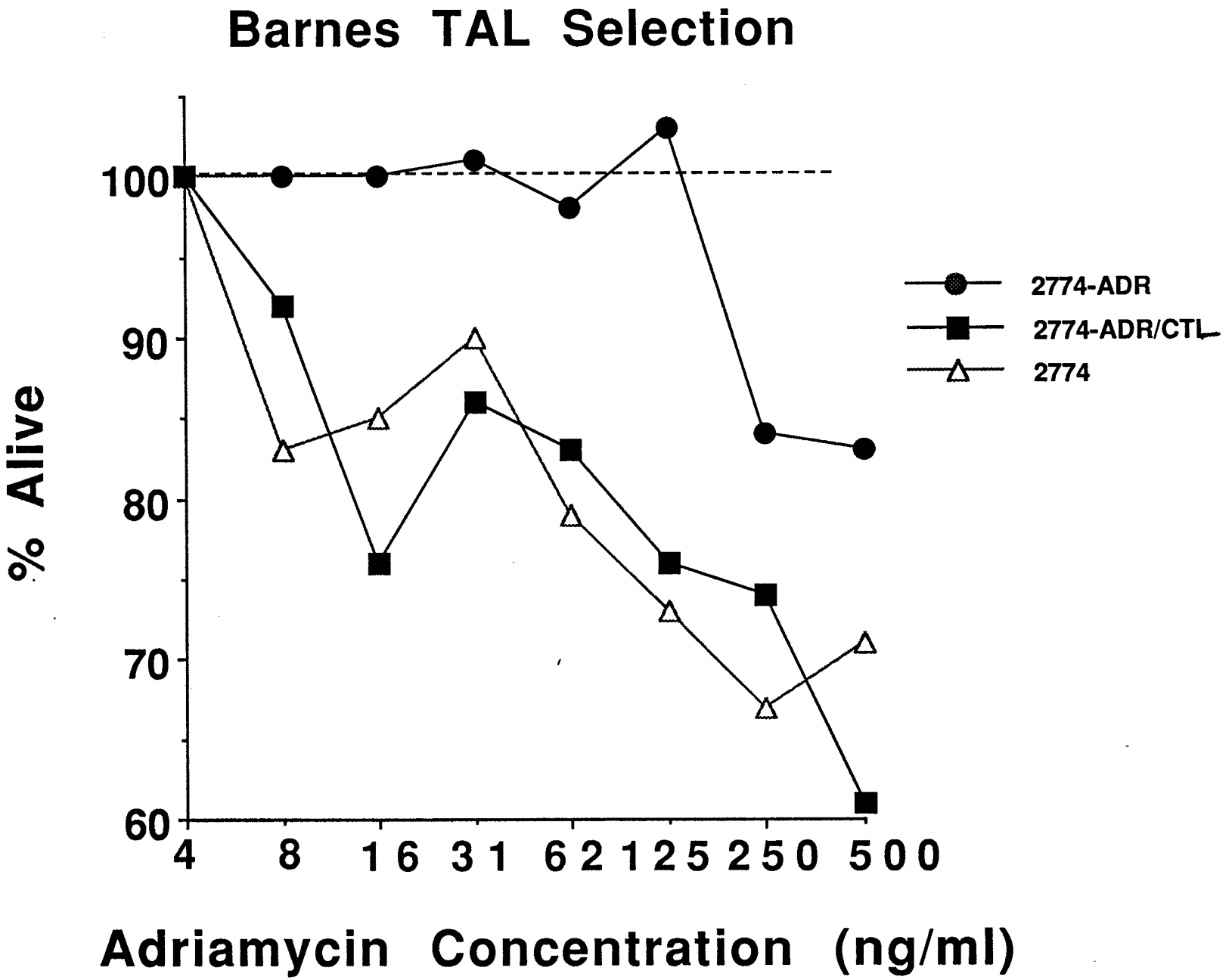

Peak 4
O.D. 220
Fraction Number

Table I. Differential Antigen Expression in Drug-Sensitive and Drug-Resistant Tumor Cell Lines.

Tumor Cell Line	Mean Level of Fluourescence	
	Drug-Sensitive	Drug-Resistant
A. HLA Class-I		
MDA 2774	155	215
SKOV3	190	216
CaOV3	194	346
MCF-7[1]	80	205
SKBr3[1]	80	120
MDA MB453[1]	44*	44*
B. HER-2/neu		
MDA 2774	153	153
SKOV3	385	396
CaOV3	10	10
MCF-7	335	320
SKBr3	139	139
MDA MB453	613	536
C. ICAM-1		
MDA 2774	230	190
SKOV3	111*	119*
CaOV3	78	99
MCF-7	275	274
SKBr3	50	60
MDA MB453	116*	119*

*Negative samples: Antibody stained population showed no difference in comparison to negative control.
[1]Indicates breast tumor lines


Barnes TAL
% Specific Lysis
50
40
30
20
10
0
2774
SKOV3
MDA-MB453
Targets
WT
DR
DR+W6/32


Barnes TAL Selection
% Alive
100
90
80
70
60
4
8
16
31
62
125
250
500
Adriamycin Concentration (ng/ml)
2774-ADR
2774-ADR/CTL
2774

[CANCER RESEARCH 57, 87–93, January 1, 1997]

Identification of Naturally Processed Human Ovarian Peptides Recognized by Tumor-associated CD8$^+$ Cytotoxic T Lymphocytes

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ABSTRACT

Identification of naturally processed peptides recognized by tumor-specific CTLs may lead to epitope-specific tumor vaccines. Because these epitopes may be expressed differently on epithelial tumors and may differ in their ability to induce CTL *in vivo*, we have isolated the HLA-A2-peptide complexes by immunoaffinity from an established ovarian tumor line transfected with and expressing *HLA-A2* gene. High-performance liquid chromatography-fractionated peptides were used to reconstitute epitopes recognized on HLA-A2 by three HLA-A2$^+$ CD8$^+$ CTL lines. These lines recognized at least three of the same groups of fractions (designated SKOV3.A, -B, and -C) but showed differences in the pattern of recognition of other fractions. To gain insight in the epitope distribution by freshly isolated ovarian tumors, we compared the recognition of peaks SKOV3.B and -C with the corresponding peaks from an ovarian tumor (OVA-6) that expressed similar levels of HLA-A2, using one of these lines (CTL-OVA-5) as indicator. CTL-OVA-5 recognized a large number of epitopes from peaks B and C rechromatographed on more resolving high-performance liquid chromatography gradients. Although a number of peaks appeared to be coincident on both SKOV3 and OVA-6, an even higher number appeared either not to overlap or to overlap only partially. These findings, which represent the first analysis of the epitopes presented by a patient tumor, suggest that the use of tumor line-derived peptides for vaccination may require selection of the epitopes corresponding to the ones presented by freshly isolated human tumors.

INTRODUCTION

The development of rational immunotherapy approaches for human cancers will depend on a detailed understanding and quantitation of the host antitumor responses. These responses involve recognition by effector CTLs of specific epitopes on malignant tissues and immunogenic epitopes, *i.e.,* peptides that can induce *in vitro* and *in vivo* antitumor responses. CTL epitopes are short peptides (8–10 amino acids long) that are presented on the cell surface by MHC class I molecules (1). Because peptides of distinct sequences can be distinguished by their physicochemical characteristics (2), elution under defined pH and concentration conditions proffers an approach to map the universe of epitopes recognized by tumor-reactive CTLs. This may allow us to establish which peptides (or groups of peptides) correspond to common dominant epitopes recognized by *ex vivo*-induced tumor-reactive CTLs. Due to T-cell receptor plasticity, the common epitopes can be reconstituted by either unique or cross-reactive peptides (3, 4). Furthermore, for CTL induction, tumor Ags[2] may be presented to T cells by professional Ag-presenting cells (5), which can process particulate Ags for MHC class I presentation (6, 7). Thus, identification of the natural peptides presented in association with MHC molecules on human tumors should be important in the development of novel approaches for tumor-specific CTL induction.

To date, with the few exceptions of melanoma Ag, gp100, and MART-1 (8–10), there is limited information on the identity and density of antigenic peptides and CTL epitopes presented by human solid tumors. The presence, distribution, and density of these epitopes on freshly isolated epithelial tumors is still unknown. Our studies as well as research from other laboratories have shown that multiple epitopes can be recognized on epithelial tumors (*i.e.,* ovarian, colon, pancreatic, breast, and lung; Refs. 11–13). Because acid-eluted peptides have been shown to be immunogenic by inducing antitumor immunity to established model tumors (14, 15), this raises the need for adequate sources of tumor epitopes for human studies. This can be accomplished with freshly isolated primary tumors only rarely because the small amount of peptides recovered preclude peptide sequence analysis. An approach to overcome these limitations is to use ovarian and breast tumor lines expressing the MHC class I molecule of interest to address the question of whether the patterns of epitope recognition by tumor-reactive CTL on the lines and freshly isolated human tumor are similar.

To address these questions, we have investigated the identity of epitopes presented by HLA-A2 on tumor targets recognized by HLA-A2$^+$ CD8$^+$ CD4$^-$ CTL lines isolated from patients with ovarian cancer. In this report, we characterized the pattern of CTL epitopes extracted from a freshly isolated ovarian tumor and HPLC separated by two consecutive gradients of ACN, and we identified common and distinct epitopes between this tumor and HLA-A2-bound peptides fractionated from an established ovarian tumor line, SKOV3. Although recognition of a number of epitopes defined as bioactive peaks eluting with distinct retention times appeared to correlate with the cell number used and the levels of HLA-A2, on these tumors for some common epitopes such a correlation was not observed, suggesting that MHC class I expression is not the only determining factor in tumor Ag expression.

MATERIALS AND METHODS

Tumor Cells and Cell Lines. The following human tumor lines were used in these experiments: (*a*) ovarian tumor line SKOV3 (HLA-A3, A28, B18, B35, and Cw5) stably transfected with the gene for HLA-A2 (16). The gene for HLA-A2 was provided kindly by Dr. William E. Biddison (National Institute of Allergy and Infectious Diseases, NIH). A tumor clone, SKOV3.A2.1E4, expressing high levels of both HER-2 and HLA-A2, has been designated as 1E4 and was selected for expansion in large numbers and peptide fractionation experiments; and (*b*) C1R:A2 cells (a kind gift from Dr. Biddison), which express only HLA-A2 on the surface, stably transfected in our laboratory with the gene encoding for the *HER-2* proto-oncogene (plasmid pCMV.HER-2 encoding a full-length HER-2.cDNA). This plasmid was a kind gift of Dr. Mien-Chie Hung (Department of Tumor Biology, M. D. Anderson Cancer Center). C1R.A2 cells were cotransfected with the plasmid SV2.Hygro (American Type Culture Collection). HLA-A2-transfected SKOV3 and C1R:A2 cells were selected with 250 μg/ml G418 (Life Technologies, Inc., Gaithersburg, MD). C1R.A2.HER-2 cells were also selected with 50 μg/ml of hygromycin B in addition to G418. This concentration was found to result in the death of more than 50% of untransfected C1R:A2 cells within 4 days in parallel

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[2] The abbreviations used are: Ag, antigen; mAb, monoclonal antibody; MCF, mean channel fluorescence; HPLC, high-pressure liquid chromatography; ACN, acetonitrile; TFA, trifluoroacetic acid; Rt, retention time; TAL, tumor-associated lymphocyte; IL, interleukin; EGF-R, epidermal growth factor receptor.

experiments. After selection with hygromycin B, C1R.A2.HER-2 cells were cloned by stringent limiting dilution. At least 20 C1R.A2.HER-2 clones (designated HER-2.A-T) were isolated, expressing variable levels of HER-2 receptor on their surface. A clone of C1R:A2.HER-2 cells designated here as HER-2.J was selected for additional studies.

Ovarian tumor cells were collected from the ascitic fluid of a patient with epithelial ovarian cancer and separated from debris and lymphocytes by centrifugation over two gradients of Ficoll as we have described previously (17, 18). They were HLA-$A2^+$ and expressed high levels of HER-2 (HER-2^{hi}). These tumor cells were designated as OVA-6.

Effector CTL Lines. T-cell lines from TALs were grown in RPMI 1640 medium containing 10% FCS and supplemented with 2 mM L-glutamine, and 100 μg/ml gentamycin (complete RPMI medium) and 50–100 units/ml of IL-2 (Cetus). Most effectors expanded, because T-cell lines contained >95% $CD3^+$ cells and variable proportions of $CD8^+$ and $CD4^+$ cells. For these experiments, $CD8^+$ cells were selected on mAb-coated plates as we described (16). The resulting cells were >95% $CD3^+$ $CD8^+$ cells. Effector CTL-3 used in these studies has been reported previously (16). CTL-4 and CTL-5 were also obtained from TALs from other patients with adenocarcinoma of the ovary. The corresponding tumors were HLA-$A2^+$ and overexpressed HER-2 (HER-2^{hi}).

Immunofluorescence. Target cells were tested in fluorescence experiments to confirm the expression of HLA-A2, MHC class I, and HER-2, as described previously (15). Hybridomas secreting mAbs BB7.2, MA2.1, and GAR-3 (HLA-A3 specific) were obtained from American Type Culture Collection. In brief, OVA-6, SKOV3, and HER-2.J cells were incubated with mAb specific for MHC class I and mAb *Ab2* specific for the extracellular domain of HER-2 (Oncogene Science) followed by FITC-conjugated goat antimouse IgG. Surface Ag expression was determined by fluorescence-activated cell sorting using a FACScan (Becton Dickinson) with a log amplifier. CD3, CD4, and CD8 Ag expression on the effectors was determined by immunofluorescence with corresponding mAb. C1R.A2 clones were designated as HER-2^{hi} when MCF for HER-2 expression was ≥40. Expression of CD18, CD11a (leukotactic factor activity 1), CD54 (intercellular adhesion molecule 1), and CD58 (leukotactic factor activity 3) was also tested the corresponding antibodies (Becton Dickinson).

Tumor Peptide Extraction. SKOV3.A2.1E4 cells were grown in 10-chamber cell factories (Nunc, Thousand Oaks, CA) in complete RPMI medium. Between 1.0 and 1.5 × 10^9 cells were obtained from one cell factory. For these studies, at least 10^{10} cells of the SKOV3.A2.1E4 cloned line were grown in batches of 1.5–2.0 × 10^9 cells. Cells were collected and washed three times with cold PBS. Furthermore, cells were lysed using the buffer described previously by Slingluff *et al.* (19) containing protease inhibitors (aprotinin, leupeptin, pepstatin, phenylmethylsulfonyl fluoride, and iodacetamide), in PBS, with the difference that 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfate 0.5% was used as a lysing agent to minimize binding to C18 columns.[3] This solution is designated here as lysis buffer. $MgCl_2$ at 6 mM and glycerol at a 20% final concentration were included in the lysis buffer to minimize the denaturation of extracted proteins. Detergent-solubilized extracts of SKOV3.A2 and OVA-6 cells were obtained after centrifugation at 40,000 × *g* for 2 h. HLA-A2.1 was isolated from the supernatant obtained from SKOV3.A2 cells by affinity chromatography on protein A-Sepharose prebound with mAb BB7.2 as described (19), except that the cell extracts were preabsorbed on protein-A Sepharose (Sigma Chemical Co., St. Louis, MO) to minimize nonspecific binding. The affinity column was washed with PBS containing 0.25 M NaCl with monitoring of the absorbance ($A_{210\,nm}$) (for peptide bond) and then eluted with 0.2 M acetic acid in 1.0-ml fractions. Fractions containing material absorbing at 210 nm were boiled for 5 min to allow dissociation of peptides from HLA-A2, centrifuged through an Ultrafree CL unit (3-kDa cutoff) (Millipore), and lyophilized. Peptides with masses <3 kDa were pooled, lyophilized, and separated by reverse-phase HPLC. This approach yields primarily HLA-A2-bound peptides and to a much lesser extent peptides associated with other MHC class I molecules.

Because freshly isolated ovarian tumors are unique specimens and the corresponding MHC-class I associated peptides are important for identification of epitopes presented by other HLA molecules, peptides were extracted from OVA-6 centrifuged lysates with 0.1% TFA following the approaches of Tsomides *et al.* (20) and Sykulev *et al.* (21). This approach has the advantage of eluting peptides with affinity high for MHC class I. Peptides of mass of 3 kDa or less were isolated by centrifugation through filters with a cutoff of 3 kDa, lyophilized, and separated by HPLC.

Fractionation of HLA-A2-bound Peptides. Tumor peptides extracted from HLA-A2.1 molecules of SKOV3.A2 cells were separated in the first dimension on a Brownlee C18 Aquapore column of 2.1 × 30 mm [pore size, 300 Å; particle size, 7 μm (Applied Biosystems, Perkin-Elmer Corp.)] and eluted with a 60-min gradient of 0–60% (v/v) ACN (Sigma Chemical Company, St. Louis, MO) in 0.1% TFA (Sigma Chemical Company, St. Louis, MO) at a flow rate of 200 μl/min using HPLC system model 1090 (Applied Biosystems). HER-2 peptides E75 (369–377), C85 (971–979), E90 (789–797), and E89 (851–859) identified in the previous studies to be recognized by ovarian CTL-3 line were separated under the same conditions. Their Rts were determined, and their resolution by these HPLC conditions was established.

For the second-dimension HPLC separation, fractions from the first dimension, including the peaks of CTL activity, were pooled in two groups, B (fractions 27–33) and C (fractions 37–45). Each group was lyophilized and reconstituted in the elution buffer corresponding to the gradient used in the second dimension (described below). Then, peptides were injected into a Brownlee C18 Aquapore column of 2.1 × 220 mm, (300 Å; 7 μm) and eluted with shallower ACN gradients. The flow rate was 200 μl/min, and fractions were collected at 1-min intervals. Peak B (fractions 27–33) were separated with a gradient of 0.1% TFA in H_2O for 0–5 min, followed by 0.1% TFA in 0–20% ACN (2%/min) for 6–15 min, 0.1% TFA in 20–40% ACN (0.5% increment/min) for 16–55 min, and 0.1% TFA in 40% ACN (designated as gradient II) for 56–60 min. Peak C fractions (Fxns 37–45) were separated in a linear gradient of 0–60 min of 30–50% ACN in 0.1% TFA thus, with an increment of ACN concentration of 0.3%/min (designated as gradient III). These gradients were selected to allow better resolution of peptides eluting in the corresponding concentration of ACN in the first dimension. For example, peptides eluting in the first dimension between 37 and 45% ACN over 8 min were separated with a gradient of 30–50% ACN over 60 min. Peptides extracted from the freshly isolated tumor OVA-6 were separated by two rounds of HPLC under identical conditions as SKOV3 peptides to allow comparison of biological activity.

Ags. Synthetic HER-2 peptides E75, G89, C85, E90, E91, E92, and the EGF-R peptide F49 (22) were prepared by the Synthetic Antigen Laboratory at the M. D. Anderson Cancer Center using a solid-phase method and purified by HPLC as described (23). The identity of peptides was established by amino acid analysis. The purity of peptides was more than 97%.

CTL Epitope Reconstitution. Reconstitution of CTL epitopes was performed using HPLC fractions from the first and second HPLC dimensions. Fifty-μl aliquots of each fraction were concentrated by vacuum centrifugation (Speed Vac) to approximately 1/10 of its original volume to remove TFA and ACN, reconstituted with water, reconcentrated, and then reconstituted with RPMI medium to the initial volume and added to ^{51}Cr-labeled T2 cells in V-bottomed microtiter plates. After preincubation with peptides for 90 min, effectors were added at 10:1–20:1 E:T ratios, and a standard CTL assay was performed for 5 h as described (15). Control wells were made with T2 cells incubated with equal volumes of the same fraction of HPLC-separated peptides without CTLs, to account for direct cytotoxicity of HPLC fractions themselves.

The percentage of specific lysis was determined from the equation $A - B/C - B \times 100$, where A is the ^{51}Cr release from T2 cells by effectors in the presence of a peptide fraction, B is the release from T2 cells in the presence of the same volume of the same HPLC fraction but in the absence of effectors, and C is the maximum ^{51}Cr release. At least two determinations were made from each fraction.

RESULTS

Similar Patterns of HLA-A2-associated Recognition for Peptides Isolated from an Established Line and a Freshly Isolated Ovarian Tumor. To evaluate the role of the transfected HLA-A2 as a presenting molecule for the ovarian $CD8^+$ CTL, we tested recognition by the ovarian CTL lines CTL-OVA-3 and CTL-OVA-5 of the SKOV3, SKOV3.A2 (1E4), C1R.A2, and C1R.A2.HER-2.J cells in

[3] V. Engelhard, personal communication.

parallel with OVA-6. SKOV3 and SKOV3.A2 cells are of identical origin. Introduction of the *HLA-A2* gene is expected to allow presentation of a number of endogenously processed peptides. Similarly, C1R.A2 and C1R.A2.HER-2.J cells are of identical origin. C1R.A2 cells express a complete and functional antigen-processing and transport system. In these cells, introduction of the *HER*-2 gene is expected to lead to the presentation of a number of peptides derived either from HER-2 or from proteins subject to upregulation or stabilization of expression by HER-2 overexpression (24). Because these CTL lines were shown previously to recognize HER-2 peptides (16), failure of the effectors to lyse C1R.A2.HER-2 cells would suggest that peptides recognized are not presented, and/or other peptides derived from HER-2 (which are not recognized) are processed and presented by the C1R.A2.HER-2 cells.

The results in Fig. 1, *A* and *B*, show that target recognition by CTL-OVA-3 and CTL-OVA-5 is associated with HLA-A2 expression. Similarly, both CTL-OVA-3 and CTL-OVA-5 recognized OVA-6 (Fig. 1). Recognition of CTL-OVA-3 and CTL-OVA-5 was inhibited by antibodies to MHC class I but not to MHC class II (data not shown).

For comparative analysis of epitope recognition, peptides were extracted from a freshly isolated ovarian tumor, OVA-6, and the clone SKOV3.A2.1E4 (designated SKOV3.A2). Both tumors expressed similar levels of HLA-A2 (SKOV3.A2; 80% HLA-A2$^+$ cells; MCF, 144; OVA-6: 75% HLA-A2$^+$ cells; MCF, 175). The levels of HER-2 were significantly higher on SKOV3.A2 cells (MCF, 324) compared with OVA-6 (MCF, 160).

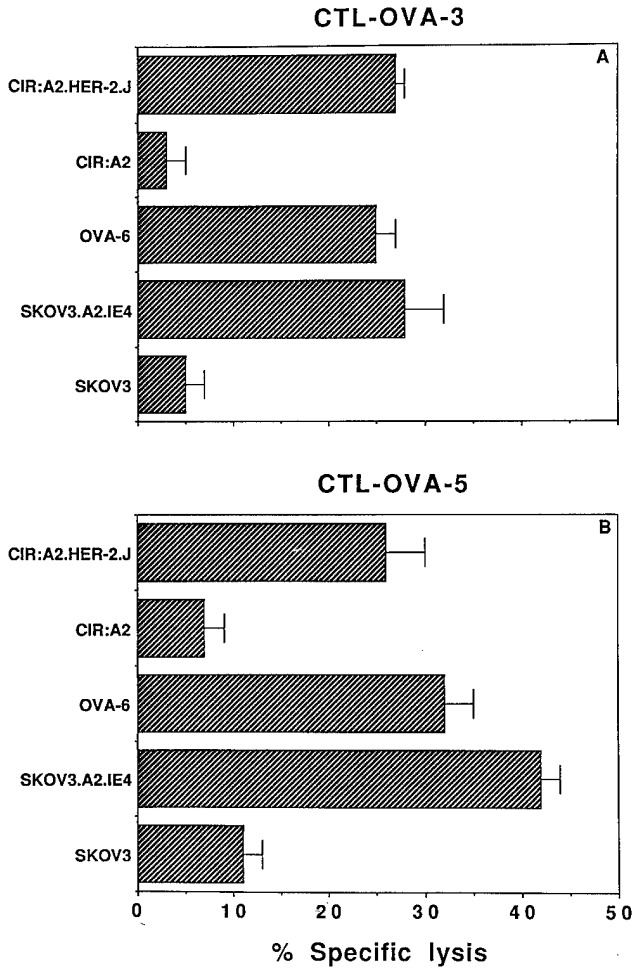

Fig. 1. Recognition by ovarian CD8$^+$ CD4$^-$ CTL lines CTL-OVA-3 and CTL-OVA-5 of the ovarian tumors SKOV3 and OVA-6 and C1R.A2 cells expressing transfected *HLA-A2* and *HER-2* genes. E:T ratio was 20:1. Results were obtained in a 5-h CTL assay.

To establish whether HLA-A2-bound bioactive peptides extracted from SKOV3 can be detected, we compared the recognition of SKOV3.A2 peptides by two ovarian CTL lines, CTL-OVA-3 and CTL-OVA-4 (Fig. 2 *A* and *B*). Equal amounts of SKOV3 peptides separated in the first-dimension HPLC were used for both CTL lines. Analysis of recognition of SKOV3.A2, fractions 10–34, by each of the CTL lines revealed four major peaks of biological activity. Two of three peaks, SKOV3 (fractions 22–27) and SKOV3 (fractions 29–32) appeared to coincide, and the third peak SKOV3 (fractions 18–21) appeared to overlap partially. The positions and heights of the peaks of more hydrophilic peptides eluting with smaller Rts were different between CTL-OVA-3 and CTL-OVA-4. This may suggest that different antigenic specificities are present in each CTL population. Furthermore, differences in the magnitude of response of CTL-OVA-3 and CTL-OVA-4 to peptides in peaks SKOV3 (fractions 22–27) and SKOV3 (fractions 29–32) were observed, suggesting not only the presence of common tumor Ag in the SKOV3 cells, but also differences in the frequency of CTL clones reacting with these peptides. The range of CTL activity observed was similar to the levels reported in recent studies for melanoma (10), and ovarian carcinoma (13).

The ascites that were the source of OVA-6 cells contained mainly tumor cells and very few lymphocytes. These lymphocytes could not be grown in sufficient numbers to perform the CTL assays. The use of high concentrations of IL-2 to promote growth lead to MHC-unrestricted lysis (data not shown). Thus, the analysis of OVA-6 peptides used established CTL lines.

Because of the limited amount of fresh tumor material, identification of bioactive peaks was made only with CTL-OVA-5. For epitope reconstitution studies, we used peptides extracted from different numbers of SKOV3.A2 and OVA-6 cells. The rationale of this approach is that if an epitope is expressed at similar densities in both tumors as shown by Griem *et al.*, the use of different amounts of peptides for epitope reconstitution would be detected as a proportional change in the CTL activity (26). SKOV3 peptides from 9.5×10^7-cell equivalents were added in each well in the CTL assay. For OVA-6, the equivalent number was 5.5×10^7 cells, resulting in a ratio of 1.8:1.0 between SKOV3 and OVA-6 peptides. This ratio was also maintained for determination of recognition of peptides separated in the second dimension. Indeed, the cytotoxicity levels detected with CTL-OVA-5 were in most instances higher with SKOV3 than with OVA-6 peptides (Figs. 2 and 3), suggesting that the epitopes detected using cell lines are the most stimulatory ones.

This should be important for peptide quantitation and epitope identification studies, because differences in CTL epitope expression have been correlated in some (26, 27) but not in other studies (3) with different levels of MHC class I expression. To determine whether a similar pattern of bioactive peptides is expressed by the freshly isolated ovarian tumor OVA-6, recognition of OVA-6 peptides separated in the first-dimension HPLC and of SKOV3.A2 peptides was tested in parallel using as effectors CTL-OVA-5 cells.

We focused on fractions with higher retention times in OVA-6, *i.e.*, the elution positions associated with a potential common tumor Ag shown in Fig. 2, and assayed every fraction in this region to optimize resolution of bioactive peaks. The results in Fig. 2, *C* and *D*, show that a broad peak of SKOV3 (fractions 20–25) corresponds to a double peak of OVA-6 (fractions 22–26); a broad peak of SKOV3 (fractions 28–34) corresponds to two resolved peaks of OVA-6 (fractions 28–29) and OVA-6 (fractions 31–32). A third broad peak of SKOV3 (fractions 39–44) corresponds to peak OVA-6 (fractions 38–41). These results indicate that CTL-OVA-5 recognizes peptides present in

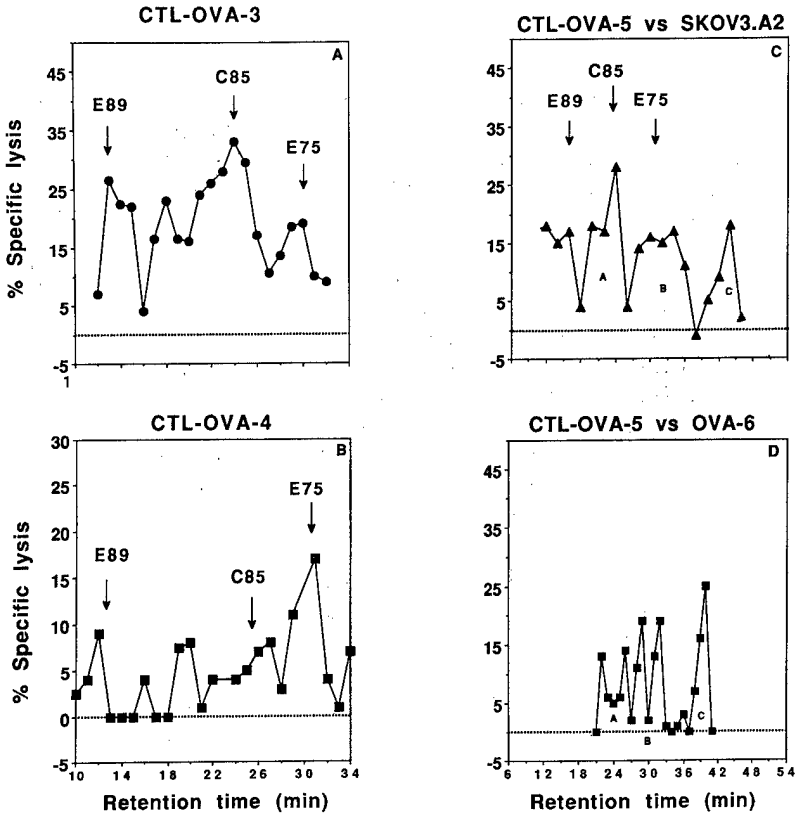

Fig. 2. *A* and *B*, recognition by ovarian CTL lines CTL-OVA-3 (*A*) and CTL-OVA-4 (*B*) of HLA-A2-bound peptides from the SKOV3.A2 cell line fractionated in the first-dimension HPLC. *C* and *D*, recognition by ovarian CTL line CTL-OVA-5 of HLA-A2-bound peptides from SKOV3.A2 cells (*C*) and of TFA-extracted peptides from the freshly isolated ovarian tumor OVA-6 (*D*). Both fractionations employed first-dimension HPLC conditions as described in "Materials and Methods." The E:T ratio was 20:1. Percentages of specific lysis of T2 cells that were not incubated with HPLC fractions were 2% for CTL-OVA-3 and 4% for CTL-OVA-4. These values were not subtracted from the cytotoxicity values induced by peptide fractions, because the control T2 cells were not incubated with column fractions. The cpms of ^{51}Cr release by T2 cells incubated with HPLC fractions in the absence of effectors were in most instances lower than those of ^{51}Cr release of T2 incubated without HPLC fractions. Details are presented in "Materials and Methods." The Rts of the HER-2 peptides separated under the same conditions were: C85 (HER-2, 971–979), 23.7 min; E89 (HER-2, 851–859), 13.4 min; E75 (HER-2, 369–377), 31.5 min; and control hydrophobic signal peptide E91 (HER-2, 5–13), 38.4 min.

overlapping peaks of activity with CTL-OVA-3 and CTL-OVA-4. These peaks are designated as *A*, *B*, and *C* in Fig. 2, *C* and *D*. Furthermore, identification of activity in OVA-6 peptide peaks eluting in the same positions with peaks A–C with a tolerance of ±1 min (1% ACN difference) suggests the possibility that a number of active tumor peptides may be common between the freshly isolated tumor OVA-6 and the SKOV3.A2 tumor line.

Multiple Epitopes Recognized by CTLs Are Presented by HLA-A2 on Ovarian Tumors. Results presented above show that a number of bioactive peaks corresponding to tumor peptides are recognized by ovarian CTL lines. To address the question of whether the epitope repertoire is composed of a limited number of peptides, fractions corresponding to bioactive peaks A–C from SKOV3.A2 and OVA-6 were subjected to an additional round of HPLC separation. To improve resolution, a longer HPLC column (of 220 mm length) of the same diameter and pore size as the one used in the first dimension was employed. Fractions separated in the first-dimension HPLC usually contain a large number of peptides (25). Because not every peptide is active, it is possible that a number of inactive peptides of higher affinity for HLA-A2 compete with the active peptide; thus, the observed CTL activity can appear lower than the activity of the fractions subjected to additional rounds of purification (25). The analysis of CTL activity presented here was focused primarily on the peptides of higher Rt eluting in peaks B and C. To afford comparisons, the CTL-OVA-5 was used as an indicator in all the assays.

With regard to peak B, which eluted as a broad peak of peptides from SKOV3.A2, and a double peak from OVA-6 (Fig. 2, *C* and *D*), fractions 27–33, were pooled, lyophilized, fractionated using a shallower ACN gradient with increments of 0.5% per min, and subjected to CTL analysis. We focused on fractions 30–47, because they eluted at ACN concentrations ranging between 27 and 35%, *i.e.*, the range in which peak B eluted during fractionation in the first dimension. The results are shown in Fig. 3 *A* and *B*. SKOV3 peptides eluted in three major peaks of activity are designated as follows: 1 (fractions 31–36); 2 (fractions 38–42); and 3 (fractions 43–47). OVA-6 peptides eluted in three broad peaks and designated as follows: 1 (fractions 35–40); 2 (fractions 40–43); and 3 (fractions 43–47). They were preceded by a small peak (fractions 32–35) that overlapped with SKOV3, peak B1. Peak B3 was almost coincident in SKOV3.A2 and OVA-6. The peak CTL values and the shape of peaks B1 and B2 of CTL activity in OVA-6 were different from SKOV3. This suggests the possibility that peptides in peaks B1 and B2 differ quantitatively and/or qualitatively in SKOV3 and OVA-6. The fact that peptides in peak SKOV3.B1 are more stimulatory than OVA-6 peptides eluting in the same positions suggests the possibility that these peptides may have been presented and were immunogenic in an earlier-stage tumor.

Peak C, which eluted as a single peak in both OVA-6 and SKOV3.A2 fractions and likely contains more hydrophobic peptides than peaks A and B, was fractionated using a shallower (0.3% increments/min) and thus more resolving ACN gradient. The results are shown in Fig. 3 *C* and *D*. Under these separation conditions, peak C resolved in at least 8 distinct peaks of both SKOV3 and OVA-6 peptides. Comparison of the plotted CTL activity over Rt shows that peaks 2 (fractions 16–19) and 3 (fractions 20–23) are almost coincident. These peaks eluted at ACN concentrations ranging between 35.3 and 37.7%. In addition peak 4 is maximal at 24 min with a broad shoulder at 25–27 min (38% ACN) but is poorly separated (or recovered) in SKOV3. This suggests that several peptides with very similar retention time endowed with biological activity are present in both OVA-6 and SKOV3.A2 cells.

The shape and peak values of the other peptides, or groups of peptides corresponding to peaks 1, 5, 6, 7, and 8, show differences between OVA-6 and SKOV3.A2. SKOV3.C.1 (fractions 13–14) and OVA-6.C.1 (fractions 11–14) elute at similar positions, but the maximal value of SKOV3.C.1 corresponds to the shoulder of peak OVA-6.C1. Similarly, SKOV3.C5 (fractions 25–29) and OVA-6.C5 (frac-

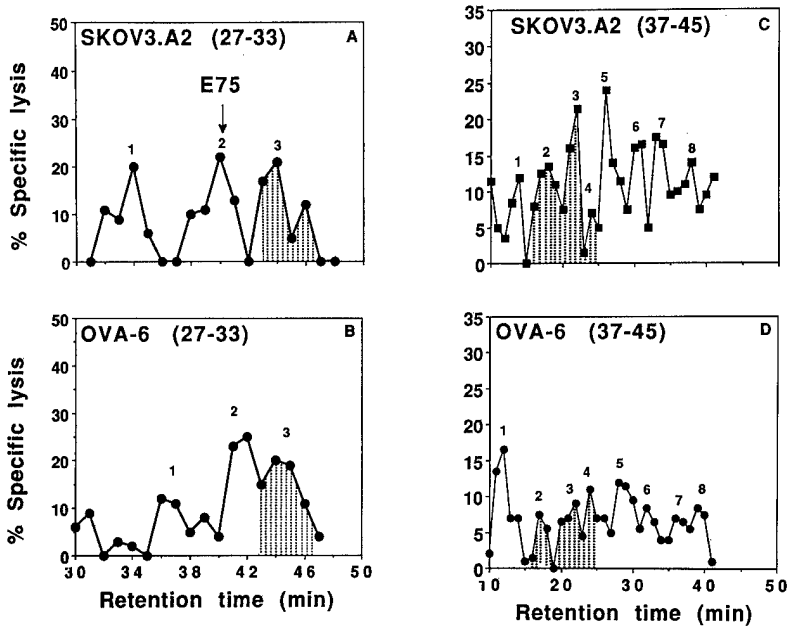

Fig. 3. Recognition by CTL-OVA-5 of second-dimension HPLC fractions corresponding to the B peaks of activity in the first dimension shown in Fig. 2. *A* and *C,* SKOV3.A2 peptides; *B* and *D,* OVA-6 peptides. *Dotted areas* indicate coincident peaks in the B3 peak of activity. The positions of elution of the HER-2 peptides used as markers for these HPLC conditions were: E90 (HER-2, 789–797), 25.4 min; E92 (HER-2, 650–658), 32.4 min; and E75, 40.5 min. The Rt of an EGF-R peptide, F49 (EGF-R, 356–364) KILGNLDFL, was 38.5 min (underlined residues indicate mutations in the E75 sequence). Lysis of T2 cells incubated with CTL-5 was as follows: no peptide (3 ± 1%); E75, 1 μg/ml (14 ± 2%); and F49, 5 μg/ml (2 ± 1%). In the same experiment, lysis of T2 cells by TAL-OVA-6 (autologous with OVA-6) was: no peptide (71 ± 10%); and E75, 1 μg/ml (73 ± 7%). TAL-OVA-6 showed slow growth. The use of high concentrations of IL-2 to enhance proliferation lead to high nonspecific cytotoxicity levels. *C* and *D,* recognition by CTL-OVA-5 of second-dimension HPLC fractions corresponding to peak C of activity in the first dimension shown in Fig. 2, *C* and *D. Dotted areas* indicate coincident peaks C2, C3, and C4. There is no lysis by peptide only if compared with control T2 cells, because the cpms released in this group were the same or lower than spontaneous release by T2 cells. The elution positions of the HER-2 peptides used as markers to verify the capacity of separation in gradient III were: F57 (HER-2, 435–443), 14.7 min; E90, 16.1 min; F49, 18.5 min; E75, 19.9 min; and E91, 29.7 min.

tions 28–30) overlap partially. On the basis of comparison of cytotoxicity values, peptides isolated from ovarian cell line appear to be more stimulatory then the peptides isolated from the fresh tumor. The reasons for the observed differences are currently under investigation. First, it should be noted that under these gradient conditions, ±1-min differences in the Rts of peaks of elution reflect differences of only ±0.3% in ACN concentration. Secondly, the amount of HLA-A2-bound peptides isolated from SKOV3.A2 and OVA-6 cells was different because of different amounts of starting material. Although we isolated a large number of OVA-6 cells, the number of OVA-6 cells was significantly lower than the number of SKOV3.A2 cells used in these studies.

To gain insight into the relationship between HLA-A2 expression and epitope density, we compared the cytotoxicity values in the bioactive peaks of SKOV3.A2 and OVA-6 that showed the highest levels of coincidence in their Rts (±1.0 min tolerance). The results are presented in Table 1. These results show that for peaks C2 and C3, the activity in the SKOV3 fractions was significantly higher than in the corresponding fractions from OVA-6. Particularly for the peak of SKOV3.C2, the activity was higher than the one expected from an 1.8:1.0 ratio (tumor line: fresh tumor). The same pattern was observed for the peaks of SKOV3.B1 and the partially overlapping peaks B2, C5, C6, and C7. For peak C4, the CTL activity was significantly higher in the OVA-6 fractions. Similarly, the levels of CTL activity in OVA-6 fractions were the same or higher in peak B3 than the levels observed with SKOV3.A2 fractions. This suggests that if for a number of tumor epitopes the density may correlate with the cell number (and with the levels of HLA expression), for others this does not apply, suggesting that they may be preferentially processed and presented by tumor cells.

Table 1 *Common epitopes on SKOV3.A2 and OVA-6 recognized by CTL-OVA-5*[a]

Peak designation (fraction No.)	Peptides							
	SKOV3.A2				OVA-6			
B3 (43–46)	18	21	5	12	16	20	19	12
C2 (16–19)	8	13	14	12	2	8	6	0
C3 (21–23)	16	22	1		8	9	4	
C4 (24–25)	6	5			17	7		

[a] Numbers indicate the percentage of specific lysis by CTL-OVA-5 of T2 cells incubated with equal volumes of the same HPLC fractions as described (26).

DISCUSSION

In this report, we present evidence that peptides extracted from an established ovarian tumor line and from a freshly isolated ovarian tumor can reconstitute the lytic activity of ovarian CTL isolated from malignant ascites of patients with ovarian cancer. Both ovarian tumors and the effectors shared HLA-A2. Assessment of the number of epitopes recognized suggests that OVA-6 and SKOV3 can present at least 11 distinct epitopes to an ovarian CTL line. Of these 11 epitopes, 4 [1 separated in gradient II (B3) and 3 separated in gradient III (C2, C3, and C4)] appear to be shared between SKOV3 and OVA-6, using as comparison factors the Rts and the shapes of the peaks. The number of shared epitopes is likely to be higher if at least some of the peptides present in the partially overlapping peaks B2, C1, C5, and C6 are identical in both samples. The Rts of synthetic peptides used as markers (Figs. 2 and 3) show that both gradients II and III are quite resolving because they can separate the HER-2 peptide, E75 (369–377), from the corresponding mutated EGF-R (356–364) peptide. Similarly, mass-spectrometric analysis of ions presumed to be peptides in peaks of gradient II and III show that each ion was present

mainly in two and no more than three consecutive HPLC fractions.[4] Thus, it is likely that most of the nonoverlapping peaks of activity correspond to different epitopes.

The total number of distinct epitopes is also likely higher, because only the epitopes eluted in two major first-dimension HPLC peaks of peptides were analyzed. Indeed, preliminary studies in our laboratory indicate that peak A can be resolved in at least five peaks of biological activity from both OVA-6 and SKOV3.A2 cells. A recent study using cultured breast and ovarian tumors reached similar conclusions regarding the potential number of CTL epitopes in breast and ovarian cancer (28). HER-2 peptides found to be active in previous studies of mapping CTL epitopes with synthetic peptides appeared to coelute with the major peaks of CTL activity. Preliminary analysis also indicates that the immunodominant HER-2 peptide (369–377) coeluted with peak B2 in gradient II. Because these CTL lines were previously shown to recognize E75, it is likely that a peptide with similar retention time forming a similar epitope on T2 cells is presented by both the established line SKOV3 and the freshly isolated ovarian tumor OVA-6.

The epitope repertoire identified is only partially overlapping. The fact that none of the effector CTLs was stimulated with either tumor suggests that the effector repertoire was not altered by the tumor epitope recognition. Two categories of epitopes have been identified: (*a*) overlapping (shared), best illustrated by peaks B2, C2, C3, and C4; and (*b*) nonoverlapping, illustrated by peak B1. With respect to the overlapping epitopes, for some, the cytotoxicity values appear to reflect differences in the cell numbers of SKOV3 and OVA-6 tumors of similar levels of MHC class I expression; for others, the distribution is uneven, suggesting that they may be preferentially processed and presented by each tumor. The latter possibility is supported by recent studies indicating that preferential expression of CTL epitopes from the ubiquitous dehydrogenase in different tissues does not correlate with the levels of MHC class I expression (3).

The variable ability of CTL-OVA5 to recognize these epitopes may derive from differential expression of the precursor of these epitopes in the fresh tumors and tumor line (10). An alternative possibility is that the decreased stimulatory ability of the fresh tumor epitopes such as peaks C2, C5, and C6 may reflect altered rates of processing of the same precursor (10, 29). This may reduce epitope generation, leading to decreased recognition by the immune system (29). The presence of nonoverlapping epitopes such as B1 is of particular interest. The fact that SKOV3.B1 is recognized suggests that such an epitope was present and stimulatory on the autologous tumor. The absence from the fresh tumor may suggest immunoselection against those cells expressing SKOV3.B1.

This study, which represents the first comparative analysis of the epitope repertoire presented by a cloned established ovarian tumor line (SKOV3.1E4) and a freshly isolated ascitic ovarian tumor, indicates that a significant number of epitopes presented by the ovarian tumor can be detected in the established tumor line SKOV3. Furthermore a significant number of the peaks of peptides eluted from SKOV3 and OVA-6 are recognized by a human breast CTL line isolated from an HLA-$A2^+$ donor, suggesting that at least some of these epitopes are shared by the breast and ovarian tumors.[5] Thus, with the possible limitations due to the use of allogeneic effectors, these results show that the potential number of CTL epitopes on breast and ovarian tumors is high, and their identification deserves additional investigation. This should be of interest for identification and characterization of tumor Ag in ovarian and breast cancer. Freshly isolated ovarian tumors are difficult to grow, and their establishment in long-term culture, to achieve the desired cell number for this type of study, usually requires specific culture conditions that involve the use of growth factors and stimulation. Thus, the comparative analysis of the epitopes using defined numbers of tumor cells fractionated under defined HPLC conditions should allow identification of bioactive peptides for tumor-associated CTLs and characterization of tumor Ag.

The possibility of characterizing tumor peptides in human ovarian and breast cancer may have important implications for understanding tumor immunity and development of epitope-specific cancer vaccines: (*a*) in contrast to melanoma, in which a large number of tumor Ags have been identified (reviewed by Boon and van der Bruggen; Ref. 30), the number of tumor Ags found in breast and ovarian cancer is significantly smaller. It comprises Muc-1 (31), HER-2 (32), the AES protein of the Notch complex (33) and possibly the folate-binding protein.[6] Identification of additional Ags may allow development of polyvalent tumor vaccines directed to several tumor epitopes. This can minimize the escape of tumor variants and establishment of metastases; (*b*) the use of HPLC-fractionated peptides from established tumor lines may allow us to focus the therapy on the immunogenic epitopes. The active peptide fractions represented less than 10% of the total peptide material eluting from each HPLC column. Their use for vaccination studies will circumvent the blocking of the presenting molecules by inactive peptides. This should increase the Ag density on Ag-presenting cells, a factor that is critical for CTL induction; (*c*) because the presence of CTLs lacking specificity in the OVA-6 tumor infiltrate, as well as the presence of large numbers of tumor cells suggest that this CTL response is ineffective at this tumor stage, vaccine strategies could be developed using these peptides for therapy of earlier stage tumors of smaller size. Epitopes from tumor lines that are recognized by CTL associated with fresh tumors may provide an unlimited source of material for induction of a therapeutic response, bypassing the limitations imposed by the small amounts of fresh tumor cells. In fact, a recent study has demonstrated the ability of acid-eluted peptides to induce curative tumor immunity (14).

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Mass-spectrometric analysis of naturally processed peptides recognized by ovarian tumor-associated CD8+ CTL

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Abstract. Antigens recognized by cytotoxic T cells (CTL) are expressed as peptides presented by MHC class I molecules. To isolate peptides from the MHC molecule HLA-A2.1 and identify epitopes that define the activity profile of ovarian CD8+ CTL, peptides were separated by reverse-phase high-pressure liquid chromatography (HPLC), and analyzed by electrospray ionization-tandem mass spectrometry (ES-MS). HLA-A2.1-bound peptides were extracted from the ovarian tumor line SKOV3 transfected with the HLA-A2.1 (clone 1E4) and C1R.A2 cells transfected with HLA-A2.1 and HER-2 (clone HER-2.J) by immunoaffinity chromatography. At least five peaks of distinct retention times (termed 1, 2A, 2B, 2C, and 3) were recognized by an ovarian HER-2^{high} (HER-2^{hi}) tumor-associated HLA-A2+, CD8+ CTL line. ES-MS analysis was performed for peak 2B peptides from both types of cells. In the four consecutive fractions of peak 2B, at least 27 and 16 ion species of mass-to-charge (*m/z*) ratio between 760-1300 were detected in 1E4 and HER-2.J cells, respectively. The abundance of four 1E4 and six HER-2.J ions believed to be peptides in four consecutive HPLC fractions in this peak matched the CTL activity profile. Of these, two ions with actual *m/z* ratios 497.3-498.4 and 792.8-793.2, were found in the peak 2B from both types of cells. Since little is known about the tumor Ag recognized in human cancers, characterization of these ions may lead to identification of novel tumor Ag in breast and ovarian cancers. This may also be useful in developing quantitative approaches to the identification of tumor Ag and the determination of epitope density on tumor and normal cells. This may help characterize the relationship between tumor immunity and epitope tolerance in human epithelial cancers.

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Abbreviations: HER-2, HER-2/*neu* proto-oncogene; HPLC, reverse phase high pressure liquid chromatography; MS, mass spectrometry; ES-MS, electrospray-ionization mass-spectrometry; TFA, trifluoroacetic acid; ACN, acetonitrile; Ag, antigen; TCR, T cell receptor; Rt, retention time; Fxn, fraction; *m/z*, mass-to-charge ratio; Da, mass unit; ESI, electrospray ionization; CID, collision-induced dissociation

Key words: natural peptides, CTL epitopes, ovarian cancer

Introduction

The development of rational immunotherapies for human cancers depends on a detailed understanding and quantitation of the host anti-tumor responses that involve recognition by CTL of specific epitopes on various normal and malignant tissues. CTL epitopes are short peptides (8-10 amino acids long) presented on the cell surface by MHC class I molecules (1). In considering peptide recognition by CTL, it is important to learn how much this recognition depends on the Ag (peptide) density on the target and on the affinity of the T-cell receptor (TCR) for the particular epitope (2,3). To address the question of epitope density, it is first necessary to identify precisely the tumor peptide involved.

This is of importance for several reasons. First, natural peptides produced by tumor Ag processing machinery may not be entirely identical to the synthetic peptides used to define CTL epitopes. The latter are usually defined with HLA-class I anchor motifs or via genetic approaches (4). Second, for human cancers, a number of peptides derived from self-proteins have been found to reconstitute the lytic function of tumor reactive CTL. Thus, with few exceptions, evidence to date reflects an immune repertoire rather than a specific and selective response to tumor (5). Third, since a number of these epitopes are derived from structurally similar hydrophobic areas (e.g. signal and transmembrane domains) of various proteins (6-8), this raises the question whether the epitope(s) defined by synthetic peptides are identical or cross-reactive with the peptides presented by the tumor. Fourth, it is still unknown whether the lack of recognition of a particular tumor by its autologous CTL reflects the lack of presentation of the tumor Ag or tolerance of the particular epitope. Tumor antigens may also be presented to T cells by professional antigen-presenting cells

(APC) (9), which can process particulate antigens or damaged cells for MHC class I presentation (10,11). Thus, identification of the natural peptides presented in association with MHC molecules on human tumors should be important for the identification of tumor Ag and consequent development of novel approaches to tumor-specific CTL induction.

To date, with the exceptions of melanoma gp100 and MART-1 CTL epitopes (12,13) there is little information on the identity and density of the peptides presented by most human solid tumors. Ovarian CTL lines were recently reported to recognize a number of HER-2/*neu* (HER-2) peptides (7,14,15), but the nature of peptides presented by the tumor is still unknown. To address the nature of Ag relevant to CTL targeting in breast and ovarian cancer, we have investigated the identity of peptides presented by HLA-A2 on targets recognized by HLA-A2$^+$ CD8$^+$ CTL lines isolated from patients with ovarian cancer. We used a cloned ovarian tumor line (SKOV3.clone 1E4) and the cloned C1R.A2 line (clone HER-2.J) transfected with HLA-A2 and HER-2, respectively. Since these cells express defined levels of MHC antigens, adhesion molecules, and HER-2, quantitation of HLA-A2-bound peptide expression can provide a reference point for analysis of peptides from freshly isolated tumors.

Materials and methods

Cell lines. The ovarian tumor line SKOV3 (HLA-A3, A28, B18, B35, Cw5) was stably transfected with the gene for HLA-A2. The gene for HLA-A2.1 was kindly provided by Dr W.E. Biddison (NIAID, NIH). A tumor clone, SKOV3.A2.1E4, expressing high levels of both HER-2 and HLA-A2 and designated 1E4 was selected for expansion in large numbers and for peptide fractionation experiments. C1R:A2 cells (a kind gift from Dr W.E. Biddison), which express the product of the same HLA-A2 gene on their surface as SKOV3.A2 cells, were transfected with the gene encoding the HER-2 proto-oncogene (plasmid pCMV.HER-2 encoding a full-length HER-2.cDNA) and co-transfected with the plasmid SV2.Hygro (ATCC). These plasmids were a kind gift of Dr M.-C. Hung, Department of Tumor Biology (M.D. Anderson Cancer Center). After selection with hygromycin B, C1R.A2.HER-2$^+$ cells were cloned by stringent limiting dilution. At least 20 C1R.A2.HER-2$^+$ clones (designated HER-2.A-T) expressing variable levels of HER-2 receptor on their surface were isolated. A clone of C1R:A2.HER-2 cells designated HER-2.J was selected for expansion in large numbers and for biochemical fractionation of HLA-A2-bound peptides.

Cell lines were grown in RPMI 1640 medium containing 10% FCS supplemented with 2 mM L-glutamine and 100 μg/ml gentamycin (complete RPMI medium). HLA-A2-transfected 1E4 and C1R:A2 cells were selected with 250 μg/ml G418 (Gibco, BRL, Gaithersburg, MD). Both SKOV3 and C1R.A2 express the same HLA-A2 gene. HER-2.J cells were selected with 50 μg/ml of hygromycin B. CTL-OVA3 effector cells used in these studies were previously described. The corresponding HLA-A2$^+$ tumor overexpressed HER-2 (HER-2^{hi}) (15). CD8$^+$ cells were isolated from cultured TAL on Ab coated plates in an AIS Microcellector (Applied Immune Sciences). CTL-OVA3 line was CD3$^+$ CD8$^+$. CD4$^+$ cells represented ≤5% of total cell population.

Flow cytometry. Target cells were tested in fluorescence experiments to confirm the expression of HLA-A2, MHC class I, and HER-2, as previously described (15). Hybridomas producing mAb BB7.2 and MA2.1 were obtained from the American Tissue Culture Collection (ATCC). Surface antigen expression was determined by flow cytometry using a fluorescence-activated cell sorter FACScan (Beckton-Dickinson) with a log amplifier. CD3, CD4, and CD8 antigen expression on the T cell lines were determined by immunofluorescence with corresponding FITC-conjugated mAb. C1R.A2 clones were designated as HER-2^{hi} when MCF for HER-2 expression was above 200 and HER-2^{low} when MCF was below 40. Expression of CD18, CD11a (LFA-1), CD54 (ICAM-1) and CD58 (LFA-3) antigens was also tested with corresponding antibodies (Beckton-Dickinson).

Peptide extraction. 1E4 and HER-2.J cells were grown in complete RPMI 1640 medium in 10-chamber cell factories (Nunc, Thousand Oaks, CA). Afterwards, cells were collected and washed three times with cold PBS. Between 1.0 and 1.5x10^9 cells were obtained from each cell factory. Cells were then lysed using a buffer containing protease inhibitors (aprotinin, leupeptin, pepstatin, PMSF, iodacetamide) in PBS (16). 0.5% CHAPS was used as lysing agent to minimize binding to C18 columns. $MgCl_2$ and glycerol at final concentrations of 6 mM and 20% were added in the lysis buffer to minimize the denaturation of extracted proteins. Detergent-solubilized extracts of 1E4 and HER-2.J cells were obtained after centrifugation at 40,000 g for 2 h. HLA-A2.1 was isolated from the centrifugation supernatants by affinity chromatography on Protein A-Sepharose prebound with mAb BB7.2 as described (16), except that the extracts were preabsorbed on Protein-A Sepharose (Sigma Chemical Co., St. Louis, MO) to minimize nonspecific binding. The column was washed with PBS containing 0.25 M NaCl. The OD_{210} nm (for peptide bond) of the eluate was monitored. The column was then eluted with 0.2 M acetic acid. The eluate was boiled for 5 min to allow dissociation of peptides from HLA-A2, centrifuged through an Ultrafree CL unit (3 kDa cut-off) (Millipore), and lyophilized. For these studies, at least 2x10^{10} cells each of 1E4 and HER-2.J were grown in batches of 1.5-2.0x10^9 cells. Peptides with masses <3 kDa were pooled, lyophilized, and separated by HPLC.

HPLC fractionation of HLA-A2-bound peptides. Tumor peptides extracted from HLA-A2.1 molecules of both 1E4 and HER-2.J cells were separated in the first dimension on a Brownlee C18 Aquapore column (2.1x30 mm; pore size, 300 A; particle size, 7 μm; Applied Biosystems, Perkin-Elmer Corporation). They were eluted with a 60 min gradient of 0-60% (vol/vol) acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA) (Gradient I) at a flow rate of 200 μl/min using Model 1090 HPLC system (Applied Biosystems) as previously described (16-18). For separation in the second dimension, pooled fractions from the first dimension corresponding to

the peak of elution of peptide E75 and the corresponding peak of CTL activity were injected into a Brownlee C18 Aquapore column (2.1x220 mm, 300A, 7 μm) and eluted on a shallower gradient: 0-5 min, 0-15% ACN in 0.1% TFA; 5-45 min, 15-35% ACN in 0.1% TFA; and 45-60 min, 35-60% ACN in 0.1% TFA (Gradient II). The flow rate was 200 μl/min, and fractions were collected at 1 min intervals.

Synthetic peptides were prepared by the Synthetic Antigen Laboratory at M.D. Anderson Cancer Center using a solid-phase method and then purified by HPLC. The identity of peptides was established by amino acid analysis. The purity of peptides was more than 97%. HER-2 peptides E75 (369-377, KIFGSLAFL), C85 (971-979, ELVSEFSRM), E90 (789-797, CLTSTVQLV), and E89 (851-859, VLVKSPNHV), which were shown in previous studies to be recognized by ovarian CTL-OVA3 cells, were separated under the same conditions using Gradients I and II. Their elution peaks and retention times (Rt) were determined.

CTL assays. CTL epitopes were reconstituted from HPLC fractions obtained in the first and second RP-HPLC dimensions (Gradients I+II). Aliquots of each fraction (50 μl) were concentrated by vacuum, reconstituted with RPMI medium to the initial volume, and then added to ^{51}Cr-labeled T2 cells in V-bottom microtiter plates. After incubation of the cells with peptides for 90 min, effectors were added at various effector/target (E/T) ratios, and a standard CTL assay was performed for 5 h as previously described (15). Control wells contained T2 cells incubated with HPLC-fractionated peptides without CTL. Percent of specific lysis was determined by the equation (A-B)/(C-B) x 100, where A is lysis of T2 cells by effectors in the presence of a peptide or an HPLC fraction, B is spontaneous release from T2 cells in the presence of the same peptide but in the absence of effectors, and C is the maximum ^{51}Cr release.

Mass spectrometry. Electrospray-ionization-mass-spectrometric (ES-MS) analyses were performed on a Finnigan MAT TSQ70 triple-quadrupole instrument upgraded with TSQ700 software and a 20-kV conversion dynode electron multiplier. The Vestec ES source (PerSeptive Biosystems, Vestec Products, Cambridge, MA) was modified as previously described by Emmett and Caprioli (20). Typical ES operating parameters were as follows: needle voltage, 3.5 kV; nozzle voltage, 250 V; repeller voltage, 10-12 V; source block temperature, 240±5°C. Standard peptides were dissolved at 1-2 nmol/μl in water or 0.5% aqueous acetic acid (V%), diluted to 10 pmol/μl with 100:100:0.5 (vol/vol/vol) methanol/water/acetic acid (ES-MS solvent), and introduced into the MS by infusion at 0.82 μl/min. HPLC fractions obtained from biological samples were first lyophilized to dryness in polypropylene microcentrifuge tubes. The dried samples were then reconstituted in either 10 μl 0.5% acetic acid and an equal volume of methanol or in 20 μl ES-MS solvent. One-microliter samples were then introduced into the mass-spectrometer by injection into the infused ES-MS solvent via a Rheodyne 8125 injector fitted with a 5 μl sample loop.

Collision-induced dissociation (CID) mass spectra (MS–MS) were obtained with argon collision gas at a pressure of 1-2.5 mTorr and a collision offset potential of -6 to -20 eV. Spectra were acquired at the rate of 1 scan/second (or approximately 1000 Da/s, depending on the mass range needed) with a running average of 16 scans. For daughter-ion scans, the resolution on the first quadrupole (Q1) was adjusted to allow transmission of ±2 Da from the center of the mass of interest. A peak width of 1 Da was used for post-acquisition spectral averaging and for quantitation by manual integration of selected ion chromatograms from the injection analyses. Data analyses were performed using Finnigan MAT ICIS software.

In certain instances, samples containing hydrophobic material were first reconstituted with 0.5% acetic acid, and then mixed with an equal volume of methanol. Peptide standards (des-Tyr^1-Leu-Enk, *m/z* 393.2 $(M+H)^+$, substance P, 1-11 *m/z* 674.4 $(M+2H)^{+2}$; ß-casomorphin *m/z* 790.4 $(M+H)^+$; and bovine insulin, *m/z* 956.6 $(M+6H)^{6+}$ were analyzed to verify the sensitivity of detection. Each sample was analyzed at least twice. Sensitivity of detection was at the level of 25 fmol/μl sample injected. Determinations did not vary more than 2-4% in two independently performed experiments (data not shown).

Synthetic HER-2 peptides E75, C85, and E92 (15) were sequenced by MS–MS. To facilitate the interpretation of results, the CID spectrum in certain instances was compared with that recorded for the corresponding methyl ester. In the spectrum of the methyl ester, signals containing the C-terminus and Asp and Glu groups shift by increments of 14 Da. Methyl esterification of carboxyl groups was performed using acetyl chloride in methanol as the methylating agent. Data for sequence reconstitution were analyzed using the program PEPSEQ version 1.2 software (21). This program identifies candidate sequences based on the concordance of determined and predicted peak values of ions in a candidate sequence. The lowest deviation between the experimentally determined versus theoretical values for the respective ions was defined as the lowest score/peak ratio (sc/p).

Results

Definition of CTL epitopes recognized by CTL-OVA3. In our previous studies we found that CTL associated with ovarian tumors recognize peptides of distinct sequence. In addition to autologous tumors, these CTL were found to specifically recognize ovarian tumor lines that expressed the Ag presenting molecule HLA-A2 (15). CTL-OVA3 recognized several HER-2 peptides including E75 as previously reported (15). Furthermore, both HLA-A2-transfected SKOV3 cells and HER-2 transfected C1R.A2 cells were lysed by cloned CTL from a $CD8^+$ $CD4^-$ line (CTL-41) that was induced by stimulation of $HLA\text{-}A2^+$ PBMC with a variant of the peptide C85 (22). This suggests that some of the epitopes recognized on these targets by CTL are similar. SKOV3 cells constitutively overexpress HER-2 but do not express HLA-A2. In contrast, C1R.A2 cells expressed only the product of transfected HLA-A2 gene. Experiments on lysis of HER-2-transfected clones of C1R:A2 such as HER-2.B, HER-2.L, and HER-2.J by highly purified natural killer (NK) cells were performed in parallel, and results indicated an inverse

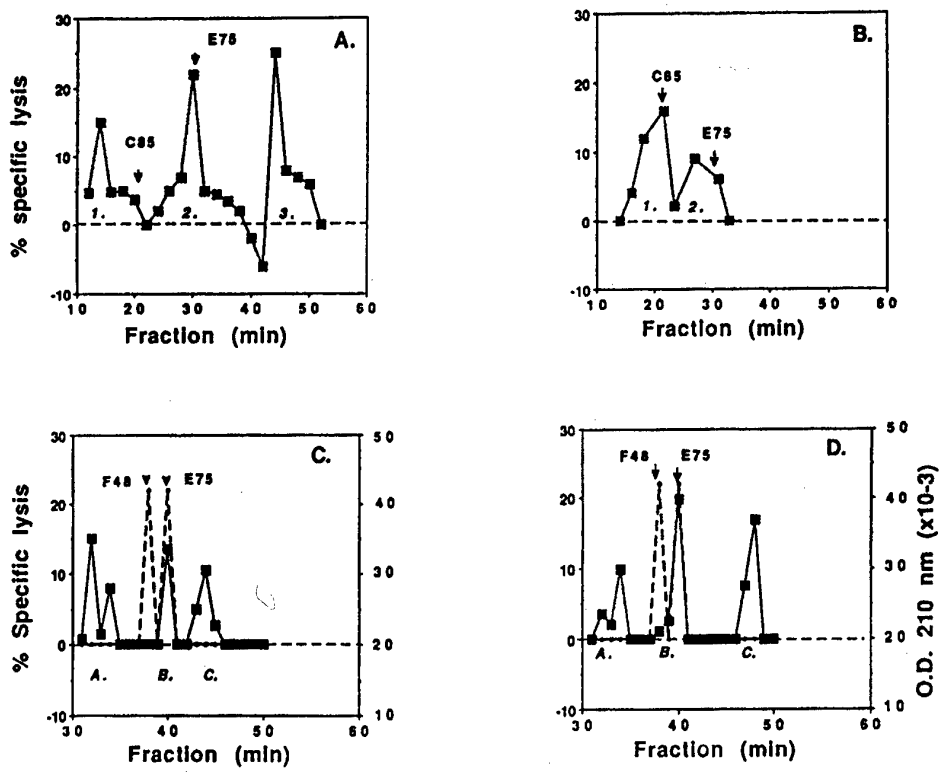

Figure 1. Reconstitution of epitopes for CTL-OVA3 from HPLC fractions containing naturally processed peptides extracted from HLA-A2.1 chains of SKOV3 clone 1E4 cells (A, C) and HER-2.J cells (B, D). Peptides were extracted as described in Materials and methods. (A) Peptides extracted from 10^9 1E4 cells separated on HPLC Gradient I using a C18 column (21x30 mm). (B) Peptides extracted from 10^9 HER-2.J cells separated on Gradient I using the same column. Peptides C85 and E75 eluted with Rt of 22.8 and 29.8 min, respectively. (C, D) Pooled Fxn 27-32 from Gradient I shown in A and B, respectively, were separated on Gradient II using a C18 column (220x30 mm). Equal amounts of peptides E75 and F48 were chromatographed individually or mixed under the same conditions as 1E4 and HER-2.J Fxn 27-32 on Gradient II using a C18 column (220x30 mm). Peptides F48 and E75 eluted with Rt of 38.0 and 41.0 min, respectively, indicating that this HPLC approach can separate mutated peptides of the same length. Effector/target ratio 20:1. (■) % specific lysis; (- -) OD_{210} nm.

correlation between HER-2 expression and susceptibility to lysis by NK cells (data not shown). These results suggest that within the CTL-OVA3 line there exists T-cell populations that recognize, (a) HER-2 peptides presented by HLA-A2 on the targets, (b) epitopes derived from proteins associated with HER-2 overexpression, (c) epitopes from proteins associated with tumor cell growth.

Recently we found that three of three ovarian CTL lines recognized naturally processed peptides eluted from HLA-A2 of the SKOV3.A2 cells. These CTL also recognized the C1R.A2.HER-2 lymphoblastoid line. Some of these peptides eluted with similar retention times with peptides eluted from a freshly isolated ovarian tumor line (23). The studies raised the possibility that common naturally processed peptides are presented by these cells, and raised novel questions on the composition of the active peaks regarding the peptide size, abundance, and identity.

To define the pattern of recognition of CTL-OVA3 for antigens (Ag) presented by HLA-A2, peptides were extracted from immunoaffinity purified HLA-A2 molecules from $1x10^9$ 1E4 and HER-2.J cells each. The presence in the peaks of HPLC fractions containing peptides with the same Rt likely indicated common CTL epitopes (16-18,22). Peptides were fractionated by HPLC on Gradient I using a C18 column (2.1x30 mm). CTL recognition was demonstrated by three distinct peaks of 1E4 peptides (designated 1, 2, and 3), respectively as indicated by lysis of T2 cells incubated with equal volumes of each fraction eluting on Gradient I (Fig. 1). Of the HER-2 peptides, C85 (971-979), eluted in Fxn 22-23 (Rt=22.8 min), while E75 (369-377) eluted in Fxn 29-30 (Rt=29.8 min), suggesting that peptide(s) of similar Rt with E75 were present in peak 2 of 1E4 peptides. Similar patterns of activity with the same HPLC fractions separated on Gradient I were observed for two other ovarian CTL lines, CTL-OVA4 and CTL-OVA5. The position of peak 2 (Fxn 28-32) was identical for CTL-OVA3, CTL-OVA4, and CTL-OVA5 cells (23).

These results suggest the presence in these HLA-$A2^+$ patients of CTL of common specificity. We focused first on the bioactive peak 2 because it was recognized by all three of our ovarian CTL lines. Since each fraction was assumed to contain a large number of peptides, the pooled active Fxn 27-32 from peak 2 corresponding to the peak of elution of E75±2 min were rechromatographed on a longer C18 column of 220 mm containing particles of the same size as in the shorter column. We used a shallower and thus more resolving Gradient II (increments of 0.5% ACN/min, between 15-35% ACN) (17,18). Fractions with higher and lower retention times than E75 were included in this separation to avoid missing shorter or longer peptides and to

minimize losses in the material due to absorption of peptides at low concentrations. The focus on peptides eluting in the area corresponding to the peak of elution of E75 was dictated by the fact that E75 was found to be immunodominant among the HER-2 peptides tested (15). Furthermore, two CTL lines recently isolated from breast tumors of HLA-A2$^+$, HER-2^{hi} phenotype (CTL-BR5 and CTL-BR6) also recognized E75 in a concentration-dependent fashion (Melichar B, *et al,* unpublished).

Rechromatography of peak 2 (pooled Fxn 27-32) under Gradient II conditions resulted in three distinct peaks of CTL-OVA3 activity. These peaks were designated 2A, 2B, and 2C, respectively. Peak 2A (Fxn 32-34) appeared as a double peak of activity. The peak 2B (Fxn 39-42) eluted around the Rt expected for E75 (Fig. 1C). To determine whether active peptides are presented by HER-2.J cells, we tested recognition of Fxn 14-33 eluting under Gradient I HPLC conditions. Recognition of peptides in these fractions by CTL-OVA3 resulted in two peaks of activity (1 and 2) (Fig. 1B) eluting with similar Rt as peptides from 1E4. Fxn 27-32 from peak 2 were pooled and separated under Gradient II conditions. The results in Fig. 1D show a similar pattern of recognition by CTL-OVA3 for HER-2.J peptides as for 1E4 peptide peaks 2A, 2B, and 2C. The Rt of E75 and of the homologous peptide from epidermal growth factor receptor (EGF-R 364-372: SISGDLHIL, designated F48) were compared under the same HPLC conditions. F48 eluted in fraction 38. E75 coeluted with the peak of CTL activity with an Rt of 41.0±0.5 min suggesting that natural peptides of similar sequence may be presented by these cells. Both E75 and F48 peptides recovered from the HPLC column were tested for recognition by CTL. E75 was active between 5-50 pmol, while F48 was inactive even at nanomolar concentrations (data not shown). These data are consistent with the presence of peptides from both 1E4 and HER-2.J cells that reconstituted three peaks of activity (2A, 2B, and 2C) for CTL-OVA3.

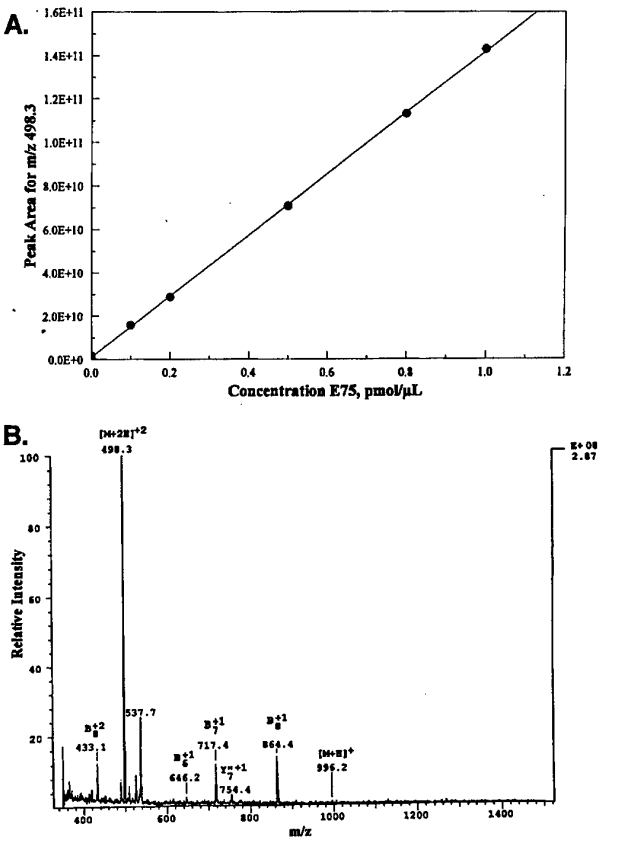

Figure 2. A, Concentration-dependent ES-MS responses of peptide E75. Synthetic peptide E75 was analyzed by ES-MS as described in Materials and methods at a multiplier setting of 1600 eV. Results are presented for the $[M+2H]^{+2}$ ion at *m/z* 498.3. The plot signal-peptide concentration in the range 10-200 fmol/1 µl sample is shown. B, Mass spectra of peptide E75 showing the double-charged $[M+2H]^{+2}$ ion at *m/z* 498.4, the singly charged $[M+H]^+$ ion at *m/z* 995.5, and a number of daughter Y and B ions (multiplier setting 1100 eV).

Mass-spectrometric analysis of peptides from HPLC fractions. To identify peptides presented by HLA-A2 by mass-spectrometry (MS), equivalent samples (in terms of cell number) were prepared from 1E4 and HER-2.J. cells. The starting material consisted of 20x10^9 1E4 cells and 10x10^9 HER-2.J cells respectively. Peptides were extracted from immunoaffinity-isolated HLA-A2 molecules using BB7.2 mAb. To maximize peptide recovery, BB7.2 mAb was added to and incubated with the eluate from the BB7.2-Protein A-Sepharose column for an additional 24 h, and the immunocomplexes were extracted after binding on Protein A-Sepharose. The recovered cell lysate was subjected to a third round of extraction using goat anti-mouse Sepharose to recover HLA-A2-BB7.2 complexes that did not bind to Protein A. However, no material absorbing at 210 nm could be recovered after this step. The peptide material separated through 3 kDa filters was chromatographed consecutively through two C18 columns (30 mm and 220 mm long, respectively) using Gradients I and II, respectively. Peak 2B from each sample was recovered in four fractions designated 39, 40, 41 and 42 (Fig. 1). Activity was found in Fxn 40 and 41 (designated active fractions) in agreement with the data shown in Fig. 1. The activities of Fxn 39 and 42 were minimal, so they were designated inactive fractions.

To establish the yields of recovery of the HPLC separation, defined amounts of peptide E75 were chromatographed through the 220 mm C18 column under Gradient II conditions and then evaluated by ES-MS. E75 recovery was estimated by quantitating the peak at *m/z* 498.3, which corresponds to the $(M+2H)^{+2}$ ion of E75, using the plot established with known amounts of E75 (Fig. 2). The yields of recovery were 88% and 83% at column loads of 5000 pmol and 100 pmol of E75, respectively (data not shown). Since both 1E4 and HER-2.J expressed high levels of HLA-A2, the total amount of peptides bound on 20x10^9 cells (assuming a minimum of 1-2x10^5 HLA-A2 molecules/cell), should be at least 3200-6400 pmol. Based on the integration of the area of peak 2 (Fxn 27-32) of the plot of absorbance at 210 nm, this peak contained approximately 10% of the peptide material collected under Gradient I conditions (data not shown). This corresponds to 320-640 pmol. This amount is higher than the amount of E75 loaded on the long HPLC column. Since the shorter HPLC column is expected to

Table I. Comparison of the amount and signal intensity of standard peptides in ES/MS.

Peptide[a]	Amount (pmol/µl)	Multiplier setting (eV)	Charge state (M+H)	Signal (ion-current) ($x10^7$)
E75	10.0	900	+1	0.01
E75	10.0	900	+2	7.5
C85	10.0	900	+1	0.16
C85	10.0	900	+2	12.0
E91	10.0	900	+1	3.8
E91	10.0	900	+2	11.0
ß-casomorphin	0.100	1600	+1	3.8
Substance P	0.100	1600	+2	11.0
Lvv-hemorphin 7	0.100	800	+1	0.22
Lvv-hemorphin 7	0.250	1600	+1	15.0
Lvv-hemorphin 7[+]	0.025	1600	+1	5.0

Peptide standards are: [a]E75, C85, and E91 are synthetic HER-2 peptides. ß-casomorphin (YPFPGPI), substance P (RPKPQQFFGLM), and LVV-hemorphin 7 (LVVYPWTQRF) are peptide standards. [b]Determined in a separate experiment.

absorb less material than the longer column, we assume that the yields of recovery from the Gradient I were at least the same with Gradient II. Thus, the recovery rate of E75 at 100 pmol is indicative of the peptide recovery in Gradient II. Therefore, we tentatively estimated the yields of HPLC fractionation at ~68% (0.83x0.83).

To obtain some indication of the abundance of unknown peptides in the HPLC fractions, known amounts of synthetic peptides were analyzed by MS, and the signal intensity was recorded (Table I). For peptides present in the range of 25-100 fmol/µl sample, better results were obtained at a multiplier setting of 1600 eV. To avoid carryover, the standards included peptides of sequences less common to epithelial cells. Several peptides were tested since the same amounts of peptides of different sequences give signals of different intensities in the ES-MS spectrum. However similar masses do not mean similar sequences, and consequently similar ionization intensities. For this reason we tested several HER-2 peptides of known mass and sequence (15). Although their signal intensities were different, the differences did not exceed one order of magnitude. Similarly the differences in signal intensities recordered for two control peptides, ß-casomorphin and LVV.hemorphin 7, did not exceed one order of magnitude. These peptides are rich in hydrophobic aliphatic residues in a similar fashion with reported CTL epitopes.

For a given peptide (e.g. E75), the signal intensity was linearly proportional to concentration (Fig. 2). Fluctuations in the linear relationship between concentration and signal intensity were observed when the concentration of E75 was high (0.5-1.0 pmol/µl). At a multiplier setting of 1600 eV, the signal intensity for 100 fmol of standard peptides was in the range of $9\text{-}20x10^7$, while for 50 fmol it was $3\text{-}6x10^7$ (Table I). For determinations at lower multiplier settings (800-1100 eV), we observed that for each increase in voltage of 100 eV, the signal intensity increased by a factor of 2. For analysis of unknown peptides from these samples, we used a multiplier setting of 1600 eV in most experiments.

Each of the peak 2B Fxn 39-42 of 1E4 and HER-2.J peptides were analyzed by ES-MS. For MS, 25% of 1E4 and 50% of HER-2 material from each of Fxn 39-42 was lyophilized and reconstituted to a final volume of 20 µl. Each determination was made from a 1 µl sample representing 2.5% of material. This would represent $5x10^8$ cell equivalents/injection. The total ion signal from equivalent samples of 1E4 and HER-2.J cells was obtained by scanning the masses corresponding to *m/z* between 200 and 1500 and then summing the obtained spectra. At each voltage, the limit of detection was defined at a signal-to-noise ratio (S/N) of 2 (24). To determine the ions present and their relative amounts (defined as signal intensity), the mass spectra of Fxn 40-41 of 1E4 in the *m/z* range of 700-1300 were determined (Fig. 3A and C). Expansion of areas from each spectrum revealed smaller amounts of additional ions. This is shown in Fig. 3B for the area 870-1100 of Fraction 40. Similar patterns of ion distribution and abundance were found in peak 2B of HER-2.J cells. This is shown for Fraction 41 (Fig. 3D). The ions shown in Fig. 3 and Table II represent an average of 2% of the total ion current, as follows: Fraction 39 (2.59%), Fraction 40 (2.17%), Fraction 41 (1.60), Fraction 42 (0.46%). Similar results were obtained for the HER-2.J peptides. The remaining current was distributed among less abundant material, which may not be composed entirely of peptides.

Twenty-seven ion species with *m/z* ratios corresponding to peptides longer than seven amino acids and an S/N >2 were found to be the most abundant in the four 1E4 fractions (Table II). Seven 1E4 ions with ratio *m/z*, of 777, 793, 807, 818, 834, 904 (actual *m/z* = 903.5-904.3), and 934, respectively and most likely singly charged were found in both Fxn 40 and 41 (Fig. 3A and C). With the exception of the ion at *m/z* 934, which was present in Fraction 39, these ions were not detected in Fxn 39 and 42. The ion at *m/z* 934

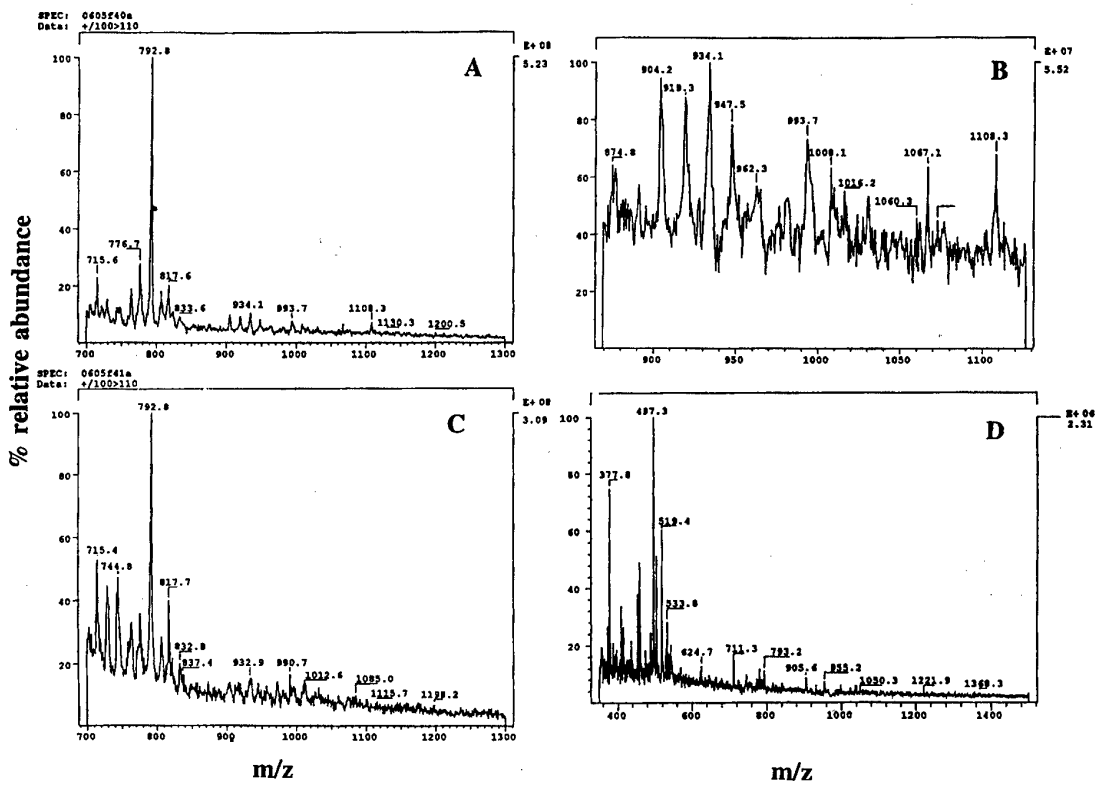

Figure 3. Identification of ion composition of peak 2B fractions by mass spectrometry. 100 µl of the Gradient II Fxn 39-42 (i.e. 50%) was vacuum dried and reconstituted in 20 µl of ES-MS buffer. Next, 1-µl volumes were injected in the electrospray ionization source. The mass-spectra of the peptides collected in each fraction were then recorded on a triple quadrupole mass spectrometer (Finnigan). Shown are chromatograms of 1E4 peptide peak 2B ions as follows: A, fraction 40, *m/z* 750-1100; B, expanded presentation of fraction 40 areas, *m/z* 860-1100; C, fraction 41; All determinations at a multiplier setting of 1600 eV. Total ion current is 52, 31, and $5.5x10^7$ for panels (A-C), respectively. (D) Mass spectra of fraction 41 (HER-2.J) showing the ions with *m/z* 775-1400 (multiplier setting of 800 eV). Note the presence and abundance of ions with *m/z* 497, 793, and 906.

was more abundant in Fraction 39 than in Fxn 40-41. The ions at *m/z* 994, 1008 and 1017 were present in Fraction 40 (Fig. 3B), but they were not detectable above an S/N >2 in Fraction 41. Twenty other ions not shown here were found primarily in each of Fxn 39 and 42.

Sixteen ion species of comparable size with 1E4 ions of *m/z* >750 were found in Fxn 39-42 of HER-2.J. Of these, five singly charged ions with *m/z* 781, 793, 906 (actual *m/z*, 905.0-905.6), 955 and 1038, respectively, were found to be most abundant in both Fxn 40 and 41 when compared with the other ions. The ions detected in Fxn 41 are shown in Fig. 3C. Their signal intensities were comparable with those of the ions detected in Fxn 40 and 41 of 1E4. The ion 454 (2+) (actual *m/z* 453.1 - 453.7) was found in both Fxn.41 and 42 (Fig. 4C and D) but not in Fxn 40, and may be the corresponding double-charged form of ion 906. The ion 497.3 was present in both Fxn 40-41 but absent from Fxn 42 (Fig. 4A-C). This ion was present in the same fractions from 1E4 cells.

Since the sequence of the ions detected was not known, a first estimation of their abundance was made by comparing their signal intensity with ß-casomorphin. Thus the ion abundance listed below represents molar equivalents of ß-casomorphin with the caveat that peptides with different sequences show variable ionization efficiencies (Table I). Thus the real abundance of these ions may be one order of magnitude higher or lower than the one determined by comparison with ß-casomorphin. Peptides corresponding to ions 807, 818, 834, 994, 1008, and 1017 detected at a similar signal intensity were present at approximately 25 fmol/µl. This equals 1000 fmol/$5x10^9$ cells or an average of 240 complexes/cell. Ion 817 was present at 65 fmol/µl in Fxn 40 and 73 fmol/µl in Fxn 41. Based on other reports, this amount should be sufficient for T-cell activation (2,25). Comparison with ß-casomorphin standards of similar mass 790 (+1), suggests that in 1E4, the ion 793 (Table II) was present at approximately 650 fmol/µl injected sample in Fraction 40, at 330 fmol/µl in Fxn 41 and at 100 fmol/µl in corresponding samples from HER-2.J cells. If the overall fractionation yield is ~50%, this represents for $5x10^9$ HER-2.J cells, 960 HLA-A2.1-793 complexes/cell. For the same number of 1E4 cells, this represents at least 5600 HLA.A2.1-793 complexes. The singly charged ion at *m/z* 497.3 was a major component of Fxn 40 and 41 from both 1E4 and HER-2.J cells but was absent from Fxn 39 and 42. Its signal intensity determined in the same experiment at 800 eV multiplier setting was for 1E4 cells 4.74 and $1.21x10^{10}$ (Fxn 40 and 41 respectively) and for HER-2.J cells $1.2x10^{10}$ and $8.2x10^8$ respectively. In a number of samples tested in separate experiments, additional peaks of similar *m/z* (i.e. 497.6 and 498.3), were found (data not shown). Because of the closeness of ion 497.3, we could not clearly identify ion

Table II. Signal strength of peptides bound to HLA-A2 from SKOV3.A2; 1E4 cells per HPLC fractions 39-42.

	Signal intensity (x10^7)[a]/Fraction No.			
m/z	39	40	41	42
767	15.6	–	–	–
772	6.9	–	–	
788	–	–	–	1.9
793	–	49.3	26.0	–
817	–	8.2	9.2	–
833	–	2.5	2.5	–
873	–	–	–	1.7
884	3.1	–	–	–
903	–	–	–	1.1
934	26.5	4.1	1.8	–
943	87.4	–	-	–
950	50.0	–	–	–
991	–	–	2.2	–
994	–	2.4	–	–
1010	11.4	–	–	
1011	–	–	2.2	–
1017	–	2.1	–	–
1045	–	–	–	1.2
1060	–	–	0.7	–
1075	6.7	–	–	–
1094	–	–	–	0.9
1197	–	–	–	1.5
1230	34.3	-	–	–
1249	85.3	–	–	–
1382	9.4	–	–	–

[a]The signal intensity for these ions was determined at a multiplier setting of 1600 eV. The ions at *m/z* 787 were identified at 800 eV in a different determination. Their signal intensity in Fxn 40 and 41 was 3.42x10^7 and 7.28x10^6 respectively. Similarly the ion at *m/z* 1008 was identified at 800 eV. The intensity of its signal in Fxn 40 and 41 was 2.4x10^7 and 6.6x10^6 respectively.

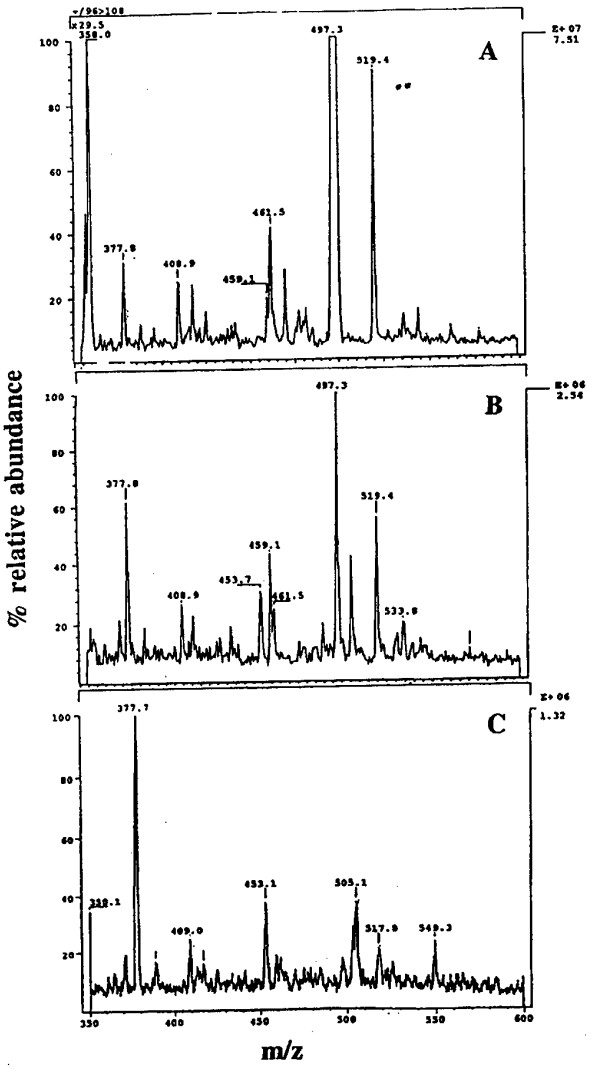

Figure 4. Mass spectra of peak 2B peptides from Fxn 40-42 of HER-2.J cells. Ion chromatograms over the *m/z* range 350-600: A, fraction 40; B, fraction 41; C, fraction 42. The ion with *m/z* 497.3 gave a saturated signal in fraction 40. Note the presence of ion with *m/z* 453 in Fxn 41 and 42. The ion with *m/z* 519.3 likely corresponds to an $(M+H+Na)^+$ ion of *m/z* 497.3 (sodium adduct). Note the absence of the ion of *m/z* 497.3 from fraction 42. Mass spectra were recorded on 2.5% of the material. Determinations were made at a multiplier setting of 800 eV. x-axis = *m/z* ratio, y-axis = relative abundance to the total ion current, which was 7.5, 0.25, and 0.13x10^7 respectively.

498.3 (2+) which would correspond to the peptide E75. The mass of the E75 synthetic peptide (498.3) is within 1 μ tolerance of the mass of the naturally presented ion (497.3). Peak 498.3 may also correspond to an isotopic form of peak 497.3 at a +1 charge state. For these reasons, the exclusive assignment of any of ions 497.3, 497.6, and 498.3 to E75, another peptide, or a lipid cannot be made based only on the *m/z* values.

Correlations between signal intensity and CTL activity of fractions in peak 2B. We compared the identity and abundance of ions in active Fxn 40 and 41 with the ions in inactive Fxn 39 and 42 of 1E4 and HER-2.J. The rationale for this approach was that correlations between presence and abundance of defined ions in consecutive fractions and the patterns of CTL recognition can focus the search for tumor CTL epitopes. For example, if an ion is present in two consecutive fractions, one active and the other inactive, the ion is less likely to be recognized by CTL if it is more abundant in the inactive fraction. Conversely, if the abundance of an ion in an active fraction is below the lower limits for binding to HLA-A2 in a T2 assay, (i.e. 10^{-12} to 10^{-13} M) it is less likely to sensitize CTL (2).

For determination of CTL activity, 10% of Fraction 40 was placed in a well of 200 μl. Of the same fraction, 2.5% was used for MS. Thus, for peptides detected at 25 and 100 fmol (as ß-casomorphin equivalents), respectively, this corresponded to 100 and 400 fmol/well (0.10 and 0.40 ng/well, assuming an average mass of 1000 Da for a nona-

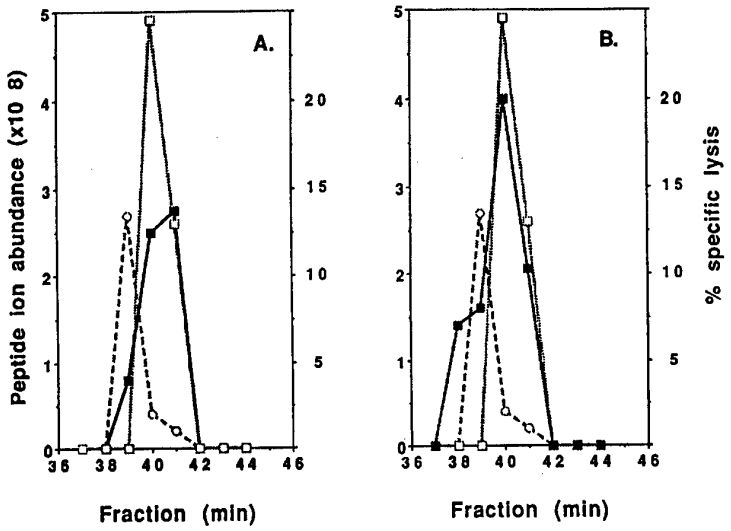

Figure 5. Identification of candidate CTL epitopes by correlation of mass spectrometry and CTL assay. Each of Fxn 39-42 contained peptides from $5x10^9$ 1E4 cells eluting from a C18 column (220x2.1 mm) on Gradient II (as described in Materials and methods). Ten percent of each sample was used in each well for cytotoxicity determinations (■). Ion abundance is shown for ions with m/z of 793 (□) and 934 (○). Ion abundance is shown as signal intensity (ion current). Scale is 10^8. A, CTL-OVA3, B, CTL-OVA5. Effector/target ratio, 20:1.

peptide) or to 0.5 and 2.0 nM, respectively. Naturally processed peptides have been reported to sensitize targets for half-maximal lysis at concentrations ranging between 0.01-50 nM (17,26,27). If any of the peptides detected at low amounts in Fxn 40 and 41 of 1E4 cells (see Fig. 3) are recognized by CTL, the TCR affinity for these epitopes should be high. This suggests that even if the actual concentration of peptides corresponding to more abundant ions (such as 497.3 and 793) is one order of magnitude lower than the one estimated by comparison with ß-casomorphin, they can be recognized by CTL with both high and low affinity for Ag.

Comparison of the activity profiles for CTL-OVA3 and CTL-OVA5 with the signal intensity of ions detected by MS indicated that the pattern of T2 lysis by CTL-OVA3 correlated with the presence in Fxn 39-42 of 1E4 ions: 497.3, 777, 904 and 793 (Fig. 5A, B). For HER-2.J peptides, the pattern of T2 lysis correlated with the presence of ions 497.3, 781, 793, 906, 955, and 1038 (data not shown). Ions of *m/z* 784, 914, and 1028 were found in both Fxn 39 and 40 but not in Fxn 41. Since their signal intensity was several-fold higher in Fxn 39 than in Fxn 40, and since the peak of CTL-OVA3 activity corresponds to Fxn 40, it is likely that peptides corresponding to these ions were not recognized by CTL-OVA3. The ion 934 was found in a significantly higher concentration (2000-3000 fmol) in Fraction 39 than in Fxn 40 and 41 in both cell lines. CTL-OVA3 showed essentially no recognition of fraction 39, but CTL-OVA5 showed low-level recognition of this fraction. Therefore, of the ion species present in Fxn 39-42 of peak 2B of 1E4 and HER-2.J, the ions 497.3 and 793 appeared to be common among SKOV3.A2 and C1R.A2.HER-2 cells. However, the CTL-OVA3 and CTL-OVA5 activity profiles for the peptide fractions in peak 2B extracted from $5x10^9$ cells did not strictly follow the abundance of ions 497.3 and 793. This suggests that in addition to 497.3 and 793, several other peptides may be candidate epitopes for CTL-OVA3 and CTL-OVA5 and may be antigenic in different individuals.

Discussion

In this study, we used immunoaffinity, HPLC separation, ES-MS, and CTL epitope reconstitution to define the pattern of possible antigenic peptides recognized by $CD8^+$ CTL in the context of HLA-A2 on ovarian tumors associated with HER-2 overexpression. Using an ovarian tumor associated HLA-$A2^+$ $CD8^+$ CTL line we established that $CD8^+$ CTL recognized at least five peaks of HLA-A2-bound peptides derived from an ovarian tumor line overexpressing HER-2. Similar peaks of activity were observed using the same effectors in C1R.A2.HER-2-derived peptides. Together these data indicate that a number of naturally processed peptides presented by HLA-A2 may be derived either from HER-2 or from proteins associated with HER-2 overexpression as previously suggested (26,27) or from proteins involved in maintenance of the transformed state. Since CTL-OVA3 cells recognize at least five distinct peaks of SKOV3 peptides, the number of CTL epitopes on ovarian tumors is likely to be higher. Indeed, ongoing fractionation studies have used peptides extracted from a freshly isolated HLA-$A2^+$ HER-2^{high} ovarian tumor (OVA-6) (23). In these studies, HPLC on three shallow gradients of ACN in the second dimension for each of the peaks 1, 2, and 3 in the first dimension indicated at least 10 peaks of bioactive peptides of distinct Rt. A number of these epitopes appear to be common for breast CTL (Melichar, *et al,* unpublished). Together, these results are in agreement with recent reports on the high number of distinct peaks of tumor peptides (in melanoma, breast, and ovarian carcinoma) recognized by CTL/TIL in the context of HLA-A2 (8,16,28).

In this study, we also established the pattern of distribution and abundance of naturally processed peptides in

active peak 2B from both target cloned cell lines. With the exception of three to four common ions of similar *m/z* ratios in each bioactive Fxn 40 and 41 of Gradient II from both cell lines, all other ions were of distinct masses. A number of peptides isolated from C1R.A2.HER-2 cells had masses identical to those of the previously reported peptides from C1R.A2 cells (24). Since the most abundant ions of mass 750-1100 in each fraction represented an average of 2% of the total ion current, this suggested that for ovarian tumor cell line SKOV3, HLA-A2 associates with a large number of different species of endogenous peptides.

In ongoing studies, we are attempting to resolve the ions in the peak 2B by determining the collision-induced dissociation (CID) spectrum and interpreting possible sequences using the PEPSEQ software. It should be noted that for all these ions, the CID spectra indicated the presence of certain impurities, likely phospholipids, that tend to associate with hydrophobic peptides, (I. Papayannopoulos and B. DaGue, personal observations). The fragment distribution is inconsistent with the existence of a single ion in this peak (either peptide or lipid) but rather with the presence of several ions in this peak. This adds to the complexity of analysis and interpretation of data toward precise sequence identification. 6 of 9 matches within 1 Da were found for B and Y ions of 497 with the values determined for the daughter ions of E75. Several possible peptide sequences were obtained with the PEPSEQ software from comparison of peak values. One of these corresponds to a peptide of sequence KXFGSXAFX (X = Leu/Ile), although the order of the amino acids in the sequence GSX could be reversed. A similar approach is taken for sequencing of the ion 793. CID spectra of the ions 454, 1008 and 1017 suggest that they represent peptides. Ions of *m/z* 1008 and 1017 corresponding to peptides 8-11 amino acids long have been detected in these fractions at significantly lower levels (1000 fmol/$5x10^9$ cells) under these experimental conditions. Ongoing studies use microcapillary HPLC and CID sequencing to resolve the components of the peak 2B. Synthetic peptides corresponding to the possible reconstituted sequences of ions 793, 1017 and 1008 are being prepared to verify the CID spectra and to determine whether these peptides are being recognized by CTL.

This raises the question as to whether peptides expressed on lower numbers of freshly isolated ovarian and breast tumors can be identified and quantitated. Although high numbers of tumor cells ($\geq 10^9$) are obtained from ascitic or advanced primary solid ovarian tumors, the amount of material available from small breast primary tumors (<2 cm), is much smaller. Since each MS determination was made on 2.5% of a sample of $5x10^9$ cells, it is likely that individual peptides of known sequence and signal in the ES-MS present at the same density, can also be detected. Therefore, peptides recovered at 100 fmol/10^8 cells can now be identified by MS, as demonstrated by previous studies with C1R.A2 cells (24) and this study (i.e., ions 1008 and 1017).

A second issue raised by identification of the naturally processed peptides is whether the amount of peptide recovered from a tumor sample is sufficient to bind HLA-A2 on T2 cells and to sensitize CTL for lysis in reconstitution assays. In other words, can the assay detect the presence of the peptide? If 10 fmol of peptides recovered are used to sensitize $5x10^4$ T2 cells in 1.0 ml (i.e., 10 0.1-ml wells, controls and samples included), this corresponds to a concentration of $10x10^{-12}$ M. Although the SD_{50} and equilibrium constant (Ka) for binding to HLA-A2 may differ among peptides, these values are in agreement with recent estimates for the minimum concentration of exogenous peptide needed to bind HLA-A2 (10^{-12}-10^{-13} M) and the number of peptides-MHC complexes required to activate CTL (2,25). Consequently, unless the peptide affinity for HLA class I and TCR is extremely high, which is the case with viral but not tumor CTL epitopes, the amount of material available is a major limiting factor in epitope identification.

Several ways to overcome these limitations are now being explored. Although direct sequencing by tandem MS of a peptide present at a level below 100 copies/cell in 10^8 cells or less may prove difficult, a novel technology for detection of peptides of known sequence at low to subfemtomole levels is available. It has been shown by Caprioli and coworkers (20,29) that by using nanoliter flow-rate desalting and preconcentration techniques with a micro-electrospray ionization source, a sample of less than 5 attomol of neurotensin methionine enkephalin can be detected using similar techniques that should allow detection and quantitating, peptides of known sequence and mass at low to subfemtomole level.

The correlations between the peptide abundance and the shape of the plot of CTL activity in consecutive fractions, does not necessarily imply that all these ions form epitopes recognized by CTL. In fact, if they are of peptidic nature, most ions with *m/z* 700-800 correspond to hepta- or octapeptides. Hepta and octapeptides were found less frequently than nonapeptides to reconstitute CTL epitopes. With respect to heptapeptides, one possibility that cannot be excluded is that they are derived from longer peptides trimmed at their ends. Another possibility is that they derive from the intracellular pool of peptides which bind a number of free HLA-A2 molecules during the immunoaffinity purification (Sette A, personal communication). Finally, a possibility that needs to be addressed is that changes in the function of the proteasome LMP2 and LMP7 proteins may lead to qualitative differences in peptide production between tumor and normal cells (30).

Identification and characterization of peptides bound to MHC class I molecules may have important implications for understanding the presentation and recognition of or tolerance to tumor Ag in human cancers. Selection of a tumor epitope for immunotherapy or gene therapy should address a number of concerns. (a) Is the epitope presented by the tumor? (b) Is its density on the tumor sufficiently high to activate effector CTL? (c) If present on normal tissues, as suggested by melanoma studies (31), is its density lower than on tumor so as to avoid CTL activation with consequent refocusing of effectors and autoimmune damage. While questions on HLA-A2 binding and TCR affinity for an epitope can be addressed with synthetic peptides and by determination of corresponding Ka and SD_{50} values, the answers to these concerns require that the epitope be identified from among MHC class I presented peptides and

then quantitated. The use of immunoaffinity would limit significantly the interference from incompletely processed epitopes (32). Therefore, approaches developed in this study should be useful for the identification and quantitation of CTL epitopes from tumors.

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Existent Proliferative Responses of Peripheral Blood Mononuclear Cells from Healthy Donors and Ovarian Cancer Patients to HER-2 Peptides*

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Abstract. *Identifying target antigens for tumor-reactive T cells is important for understanding the mechanisms of tumor escape and developing novel anticancer therapies. To date, mainly CTL responses from tumor infiltrating/ associated lymphocytes (TIL/TAL) to peptide antigens have been investigated in ovarian cancer. In the present study, the ability of self-peptides derived from HER-2/neu proto-oncogene product (HER-2) to stimulate proliferation of PBMC from healthy donors and ovarian cancer patients has been assessed. Peptide sequences from HER-2 containing anchors for major human MHC-class II molecules have been identified. These peptides induced proliferative and cytokine responses at higher frequency in healthy donors than ovarian cancer patients. Four HER-2 peptides corresponding to positions: 396 - 406, 474 - 487, 777 - 789, and 884 - 899 were able to stimulate proliferation of a larger number of healthy donors than three other distinct HER-2 peptides 449 - 464, 975 - 987 and 1086 - 1098. The pattern of responses of twenty five ovarian cancer patients was different from that in healthy donors. T cell lines were developed by stimulation with peptides from PBMC of an ovarian cancer patient who showed a stable response to all four HER-2 peptides for over six months. Each T cell line was different in its ability to secrete IFN-γ and IL-10. These results demonstrate (a) that self-peptides from HER-2 can stimulate expansion of T cells in both healthy donors and ovarian cancer patients, and (b) the ability of different peptides to stimulate secretion of different cytokines from lymphocytes of ovarian cancer patients. These results may be important for understanding the mechanisms of tolerance and autoimmunity in human cancers.*

Abbreviations: Cytotoxic T Lymphocytes, CTL, Position, P; T cell receptor, TCR; HER-2/neu proto-oncogene, HER-2; stimulation index, S.I.; standard deviation, SD, TT, tetanous toxoid.

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Key Words: HER-2, $CD4^+$, epitope, ovarian cancer, Th1, Th2, cytokines.

The HER-2/neu proto-oncogene product (HER-2) is the target of autoantibodies in breast cancer (1) and of cytotoxic T lymphocytes (CTL) in ovarian, breast, and lung cancer (2-5). Since these auto-antibodies are specific for the native conformation of the HER-2, they must be induced by the native molecule. HER-2 is present in both healthy individuals and cancer patients. Similarly HER-2-reactive CTL are specific for a number of epitopes (2-5) of which one HER-2, 369-377 was found to be immunodominant in our studies (5). These CTL were isolated from lymphocytes associated with ovarian tumors in patients with advanced disease after culture in the presence of IL-2. This suggests that $CD4^+$ T cells capable of helping either B cells, or $CD8^+$ CTL, or both may be present in the cancer patients. This raises the question as to whether CD4+ T cells capable of recognizing epitopes from human HER-2 are also present. Previous studies have

Table I. *Sequences of HER-2 peptides.*

PeptideCode	Position	Sequence[a]
F11	5- 19	A L C R W G L L L A L L P P G
D122	396 - 406	Q L Q V F E T L E E T
F12	449 - 464	G I S *W* L G **L** R S **R** E **L** G S G L
F14	474 - 487	T V P *W* D Q *L* F *R* N P H Q A
F7	776 - 789	G S P *Y* V S **R** L **L** G I C L
F6	777 - 797	G S P *Y* V S **R** L *L* G I C L T S T V Q
F8	832 - 851	G M S *Y* L E D V R L V H R D L A A R N
F10	975 - 997	F S R *M* A R S P Q R *F* V V **I** Q N **E** D **L** G
D100	1086-1098	F D G D L M G A A K G L
F13	884 - 899	V P I K *W* **M** A L **E** S **I** L R R R F

[a]Potential DR4 anchors that distinguish between DRB1*0401/0404 and DRB 1*0402 are shown in bold. Tyr (Y) and Trp (W) residues characteristic of the P1 anchors for DRB1*0401 and DRB*0101 binding motifs (21) are italicized. Prolines for protection from proteolysis are underlined. Peptide F10 extends a potential helper epitope after the CTL epitope C85 (the sequence is underlined). Both DR4 allotypes (DR4.1 and DR4.2) accept peptides with Leu, Ile, Met, Phe, and Val as P1 anchor residues (19).

shown that $CD4^+$ T cells with specificity for HER-2 can be identified in breast cancer patients (1). The extent and the existence of autoreactive T cell repertoire to HER-2 in both healthy humans and ovarian cancer patients has not been previously identified. To assess the specifIcity of these T cells and identify potential targets for epitope-specific immunotherapy, we investigated the responses to HER-2 of a group of patients with ovarian cancer subsequent to chemotherapy and a group of healthy individuals.

To characterize the T cell response to HER-2 we assessed a number of T cell epitopes of HER-2 with a set of synthetic peptides based on the HER-2 sequence. We wanted to identify a set of such peptides to which healthy donors and ovarian cancer patients respond by proliferation and determine the frequency of these responses. We stimulated PBMC from twenty five ovarian cancer patients and fourteen healthy donors with synthetic HER-2 peptides. PBMC from each donor were stimulated individually with each peptide, but not with pooled peptides. The general pattem of response was characterized by a group of four HER-2 peptides designated as D122:HER-2,396 - 406, F7:HER-2:777 - 789 F13:HER-2,884 - 899, F14:HER-2, 474 - 487 which induced a signiflcantly higher frequency of responses than the other three HER-2 peptides designated as F10:HER-2,975 - 997, F12:449 - 464, D100:HER-2,1086 - 1098 in both healthy donors and ovarian cancer patients. The frequency of responses to most HER-2 peptides was significantly lower in ovarian cancer patients who had received chemotherapy than in healthy donors.

T cell lines were raised against individual HER-2 epitopes represented by peptides F7, F13, F14 and D122 from PBMC of an ovarian cancer patient by restimulation with HER-2 peptides and expansion in IL2. Thes̨ T cell lines showed a different pattern of IFN-γ and IL-10 production. F13 induced T cells secreted significantly higher amounts of IFN-γ than IL-10 while F7 and F 14 induced T cells secreted significantly higher levels of IL-10 than F13 and D122 induced cells.

Materials and Methods

Cells. Peripheral blood nononuclear cells (PBMC) were obtained from fourteen healthy donors and twenty flve ovarian cancer patients. All patients had advanced disease. After initial surgery, they were treated with platinum (cisplatin or carboplatin). One patient was receiving primary platinum based chemotherapy. The other twenty four patients had received additional chemotherapy. The latter was either carboplatin reinduction, salvage therapy with paclitaxel or experimental therapy with several different drugs. Blood collection was made at least three weeks after the last chemotherapy administration. PBMC were isolated from heparinized peripheral blood as described (1,6). At the time of the assay the patients were not receiving chemotherapy.

HER-2 epitopes selection. Peptides to be tested in the proliferation assays were selected based on the T cell sites in HER-2 predicted by the

computer program ANT.FIND.M, the general binding motif for human class MHC-II antigens, and the presence in the sequence of anchors for a number of MHC-class II antigens: HLA-DRl, HLA-DR3, HLA-DR4, HLA-DRll, and HLA-DQ7 (7-13). The sum of the allelic frequencies of these antigens cover approximately 100% of the Caucasian and Hispanic populations and between 75 - 92% of the African American and Asian populations. For example HLAD97 is present at 28% (Caucasians) 23% (African Americans), and at 43% (Hispanics). Similarly, each of HLA-DR1, DR3, DR4 and DR11 is present between 17 - 20% in each of the major population groups. The general binding motif for various human MHC-class II molecules consist of a position 1 (P1) anchor, *i.e.* an aromatic or large aliphatic residue in the first 3 - 5 amino acids close to the N-terminus, and other major but less essential anchors at positions 4, 5 - 7, and 9 counting from the Pl anchor (13). A large number of "promiscuous" peptides are capable of binding to many different MHC-class II molecules (13, 14), because their sequences contain overlapping binding motifs for MHC-class II molecules (13, 14). The search for specific anchors for these major MHC-class II antigens in the HER-2 sequence indicated significantly more candidate epitopes for binding to HLA-DR1 (n = >20) than for HLA-DR3 (n = 11), than for binding to HLA-DR4, HLA-DQ7, and HLA-DR11.

Ten HER-2 peptides 11 - 22 residues long (Table I) were synthesized by the Synthetic Antigen Laboratory of M.D. Anderson Cancer Center, using a solid phase method as previously described (2). The identity of peptides was determined by amino acid analysis. The purity of peptides ranged between 93 - 97% as determined by HPLC. Peptides were dissolved in PBS, aliquoted at 2 mg/ml and stored frozen at -20°C until use. The codes used to identify these HER-2 peptides in this paper were assigned by the Synthetic Ag Laboratory. To ensure better representation of different binding motifs for these MHC class II antigens, at least two peptides were synthesized containing anchors for each of HLA-DR1, -DR3, and -DQ7. When possible the sequences were selected to contain anchors for two MHC class II antigens (Table I). Each of the peptides synthesized contained at least two of three anchors for each HLA-DR antigen, as shown in the Table I, and the main Pl anchors for most class II alleles. In peptides D122, F12, F7, F6, F8 and F13, positions P4 and P5 are occupied by hydrophobic, aromatic followed by aliphatic residues in that order to facilitate peptide binding in different frames.

The binding motifs of synthetic peptides for MHC-class II molecules may differ from natural ligands because the latter incorporate constraints of processing in addition to binding requirements. For these reasons, peptides were synthesized by following, when possible, the common motifs for all MHC-class II molecules defined by pool sequencing of naturally processed peptides (7). This was possible for four peptides, F6, F7, F13 and F14. In these peptides, the sequence was extended to include Pro (P) N-terminal to either the Tyr (Y), which is the P1 anchor for HLA-DR1, DR3, DR4, and DQ7, or Trp (W) which is reported by the Pl anchor for HLA-DR4 and DR1 1. Peptides F6 and F7 overlap in the first thirteen residues. In F6 the sequence was extended at its C-terminal to incorporate a region 783-797 previously reported to induce proliferation of PBMC from breast cancer patients. The sequence of F14 was also extended to include Pro at the C terminus, after Arg (R), the third anchor in the correct position for HLA-DR3 and HLA-DR1 1. Sequences were also extended at the N- and C-termini. This was made to facilitate the natural proteolytic trimming of peptides since most aminopeptidases stop cutting one residue before reaching a Pro residue (7).

Stimulation and propagation of T cells. Freshly separated PBMC from healthy donors and ovarian cancer patients were stimulated with each HER-2 peptide at a final concentration of 50 μg/ml and cultured at 1×10^6 cells/ml in RPMI 1640 medium (GIBCO) with 5% pooled human AB serum and antibiotics (complete RPMI medium) (1). After 3 - 4 days of stimulation with each peptide, cultures were expanded with IA-2 (20 - 40 U/ml) for the following week (15, 16). To induce antigen specific T cells, the cells were then "rested" for 3 - 4 days by culture in

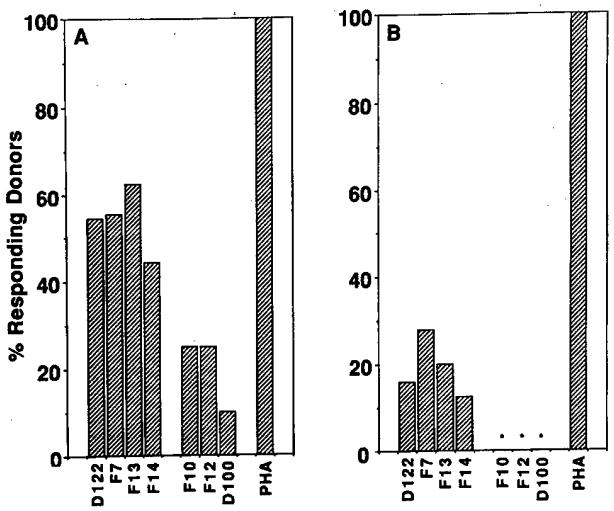

Figure 1. *Histograms of positive blastogenic responses of PBMC from (A) 14 healthy donors and (B) 25 ovarian cancer patients to seven HER-2 peptides of the sequences listed in Table I. The Y axis indicates the fraction of donors with a positive response to each peptide. A blastogenic response was considered significant when the p values for peptide stimulated vs. control cultures were <0.05. Stimulation indexes (S.I.) were obtained by dividing the means of cpm proliferation of PBMC in the presence of peptides with the means of cpm proliferation in the absence of peptide. S. I. for the peptides inducing significant proliferation ranged between 1.5- 3.0, while for the peptides that did not induce significant proliferation ranged between 0.9 - 1.2. Proliferation values (cpm + SD) are shown in Figures 2 and 3. Peptides F7 and F13 elicited the most positive responses in PBMC from both healthy donors and patients (at least 5 donors positive in each group). In (B), (*) the frequency of responses to F10, F12, and D100 respectively were as follows: F10: 1/25, F12, 1/25 and D100: 2/25.*

the absence of IL-2. Then, the cells were stimulated at a 1:1 (stimulator:responder ratio) with irradiated (10.000 Rad) autologous PBMC, which had been flrst stimulated with PHA, expanded in IL-2, and then were pulsed with individual peptides for at least 90 min at 37°C before addition to the cultures as described (16). Control cultures were stimulated with the same number of IL-2 expanded PBMC in the absence of peptides. For further expansion, four to five days later 20 - 40 U/ml IL-2 was added to the cultures for seven additional days.

Proliferation assays. The proliferation assay was done by culturing 2×10^5 PBMC from each donor in quadruplicate in a 96 well plate in 200 μl with each peptide at 50 μg/ml, with tetanus toxoid at 5 μg/ml and PHA (GIBCO) at a final concentration 1:100 for 96 h as described (1, 16). For the last 16 h, 1 μCi [^{3}H]-Tdr was added to the cultures. Afterwards, the cells were harvested and the radioactivity counted in a Beckman LS3501 liquid scintillation counter as previously described (6). A significant proliferative response was deflned as a statistically significant increase in the cpm proliferation in the tetraplicate cultures stimulated with any of the peptides, PHA, or TT, above that in cultures from the same donor that received peptide. Values obtained for cpm (^{3}H-Tdr incorporation) by the PBMC incubated with PBS, PHA or synthetic HER-2 peptides were examined by the Student's t test. Differences were considered significant when the P values were < 0.05.

Flow cytometry. T cells were tested in fluorescence experiments to determine the surface antigen expression as previously described (2, 6).

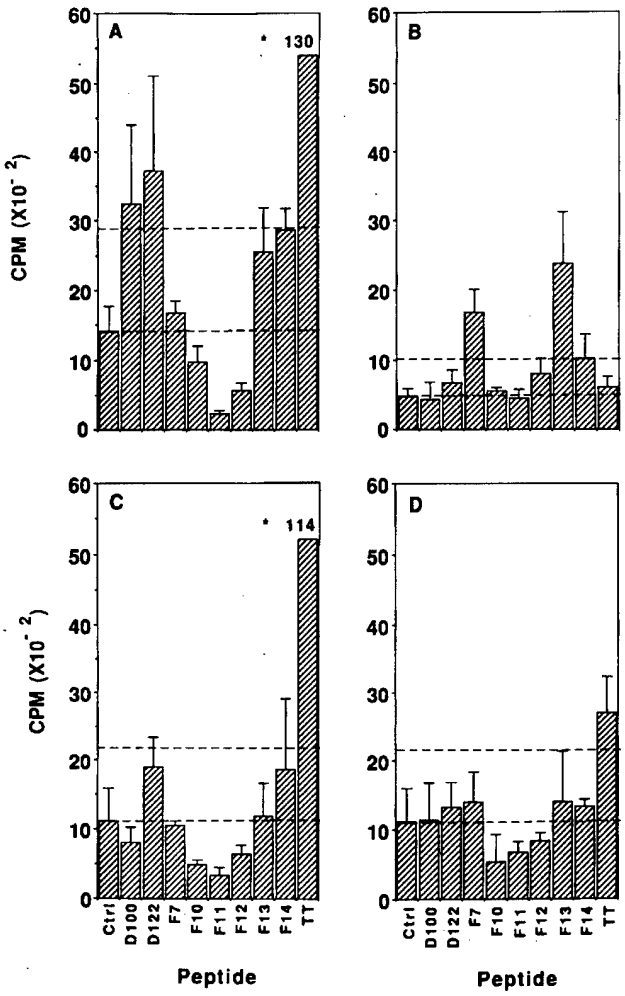

Figure 2. *Proliferative responses to HER-2 peptides by PBMC from four healthy donors A, B,C,D determined in the same experiment. Two responded to peptides as follows: Donor A to D 100, D 122, F13 and F14; Donor B to F7 and F13. Two were non-responders (panels C, D). Results indicate cpm ± SD. Dotted lines indicate proliferation corresponding to S.I. of 1.0 and 2.0 respectively. *cpm proliferation to TT not on scale.*

Surface antigen expression was determined by FACS analysis using a FACSscan (Beckton-Dickinson, Sunnyvale, CA)) with a log amplifier. CD3, CD4 and CD8 antigen expression on T cell cultures was determined by immunofluorescence with corresponding mAb FITC-conjugated (Beckton Dickinson).

Cytokine production. The ability of PBMC to produce antigen induced IFN-γ and IL- 10 was determined by culturing the PBMC either as unstimulated or stimulated with the corresponding peptides or PHA, (GIBCO) diluted 1:100, or tetanus toxoid. Supematants were collected after 48h and stored frozen at -20°C until assayed for cytokine level. Cytokine containing supematants from the T cell lines were generated by adding OKT3 and phorbol myristate acetate (PMA) to the cells to 96 well plate for 18h as described (17, 18). Afterwards, supernatants were collected for measurement of IFN-γ and IL-10 levels. IFN-γ and IL- 10 were measured by double sandwitch-ELISA using the corresponding kits provided by BioSource International (Camariyo, CA). Supematants from tetraplicate wells were pooled and tested in triplicates. The cytokine assays were calibrated with human recombinant IFN-γ and IL-10 to detect each cytokine in the range of 10-1000 pg/ml.

Results

Proliferative responses of PBMC to HER-2 peptides. To map the HER-2 peptides most frequently recognized and to identify potentially immunodominant epitopes, we determined the responses to individual peptides of PBMC from twenty five ovarian cancer patients. Patients previously treated with chemotherapy were allowed on this study, since immunotherapy approaches to ovarian cancer are usually initiated after conventional therapies thus making the responses of these donors more likely to reflect the responder status of candidates for tumor-vaccine therapies. Responses of PBMC from healthy donors to the same peptides were tested in parallel in the same experiment. Depending on the number of PBMC available from each ovarian cancer patient a minimum of four peptides and PHA were tested in the same experiment. Results are summarized in Figure 1. Peptides Fl 1 (HER-2, 5-19) (which corresponds to the highly hydrophobic signal area) F6 (HER-2, 777-797) and F8 (HER-2, 832 - 851) were not easily solubilized in PBS, thus their use for stimulation was discontinued after several assays. PBMC from most donors were tested with at least six peptides in each experiment. Each donor responded to some peptides but not to others. The lack of a common response it is not entirely suggesting that these peptides do not bind MHC molecules. A negative response of any of these peptides could also reflect T cell unresponsiveness (tolerance) to this epitope.

To ensure that the lack of responsiveness of PBMC to HER-2 peptides did not reflect a generalized suppression of responses to antigen or mitogen, all patients' lymphocytes were tested for their ability to respond to PHA. Of 29 patients tested, 28 showed significant responses to PHA compared with unstimulated cultures. All PBMC samples from healthy donors showed significant responses to PHA (data not shown). Together these results indicated that most PBMC from the ovarian cancer patients with advanced disease respond to PHA after chemotherapy and that their ability or failure to respond to HER-2 peptides did not reflect their ability to respond to a T-cell mitogen.

The frequency of responses of healthy donor PBMC to four peptides: D 122, F7, F13 and F14 was higher than the frequency of responses to three other peptides, F10, F12, and D100. The same pattem of preferential responses to F7 and F13 was seen in PBMC from ovarian cancer patients (Figure lB). The results also indicate that PBMC from ovarian cancer patients responded at a significantly lower frequency than healthy donors to F7, F13, F14 and D122. However, the decrease was not the same for all peptides. For peptides D122, F13, and F14 the decrease in responses in PBMC from patients versus healthy donors was in the range of 70%, while for peptide F7 the decrease was only 50%. No significant

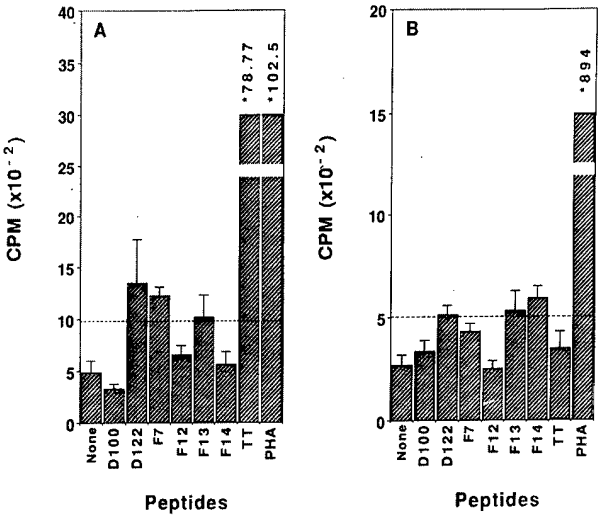

Figure 3. *Proliferative responses to HER-2 peptides of PBMC determined in the same experiment. (A) ovarian cancer patient; (B) healthy donor. Significant differences between peptide stimulated and no peptide cultures were observed in (A) for D122, F7 and F13 and in (B) for D122, F13 and F14. Dotted lines indicate proliferation corresponding to S.I. of 2.0.*

responses to peptides D100 (1086 - 1098), F10 (975 - 987), and F12 (449 465) were observed. Representative results from PBMC of four healthy donors (two responders and two non-responders) are shown in Figure 2. Donors A and B were considered responders based on the ability of their PBMC to proliferate to at least one of the eight HER-2 peptides tested. These responses were significantly higher than responses by PBMC that have not been pulsed with peptides. In both responders and nonresponders, the responses to HER-2 peptides did not correlate with the ability of the same PBMC to respond or with the magnitude of response to TT (Flgures 2 and 3). Of twenty flve ovarian cancer patients tested, seven responded to F7 with statistically signiflcant differences in cpm proliferation between peptide-induced and control cultures. Of seven F7 responding patients three responded to F13 two responded to F7, F13 and F14; one to F7 and F14; and four only to F7. Of five F13 responding patients two did not respond to F7 or Fl4. Only two patients responded to all four peptides: F7, F13, F14 and D122. There was no signiflcant difference in the magnitude of their proliferative responses to these four peptides.

For comparison, the pattem and the magnitude of the proliferative responses to HER-2 peptides of PBMC one of these patients (designated as Patient A) is shown in Flgure 3 together with those of PBMC from another healthy donor tested in the same experiment. Both donors responded to D122, F7, F13 and F14 but failed to respond to D100 and F12. To establish whether the pattern of responses to the HER-2 peptides in PBMC from ovarian cancer patients changed over time, responses to the same peptides were determined at two additional time points over five months when the patients were not receiving chemotherapy. PBMC from Patient A showed a constant high level of responses to at least two of the four peptides tested, but PBMC from a nonresponding patient failed to proliferate above the control levels. The same PBMC showed significant responses to PHA. It should be mentioned that sixteen months after the first determination, PBMC from the same Patient A responded primarily to F13 also less strongly to F7 (data not shown). PBMC from the same responding patients were tested again after several months when the disease progressed. At this time significant proliferative responses to HER-2 peptides were not observed. All three patients that responded to both F7 and F13 had stable disease. This suggests that disease progression affects the ability of PBMC from cancer patients to respond to HER-2 peptides.

In vitro expansion and cytokine production by HER-2 peptide stimulated T cells. To establish whether HER-2 peptide stimulated lymphocytes can be expanded in culture as T-cell lines, PBMC from Patient A were selected for these experiments. These PBMC were chosen because they showed a stable and significant proliferative response to at least four HER-2 peptides of distinct sequence. Primary cultures with F7, D122, F13 and F14 were initiated for four days, after which IL-2 (40 U/ml) was added for four more days. Afterwards, IL-2 was removed and the cells were "rested" in complete RPMI medium in the absence of IL-2 for four days. Afterwards, each peptide initiated culture was restimulated with autologous T cells from PHA-stimulated PBMC expanded in IL-2 prepulsed with the corresponding peptide. Control cultures were then restimulated with PHA blasts in the absence of peptides. After four days, IL-2 (20 U/ml) was added to the cultures for 48 h. The S.I. were determined by comparing the 3H-Tdr incorporation during the last 16 h. As shown in Figure 4A all peptide stimulated cultures showed an increase in S.I. of >2.0 over the control cultures. Similarly, cultures stimulated initially with TT, PHA and OKT3 in the presence of IL-2 showed a significant increase in proliferation over the peptidestimulated cultures.

To address whether T cells induced by one of the peptides can be stimulated to proliferate by another HER-2 peptide, we investigated the response of F13-induced T cells to F13, D122, F14, and F7. The results are shown in Figure 4B. PHA and OKT3 mAb were used for stimulation as positive controls. Cells were counted after four days. A signiflcant increase in number over control cultures was observed in F13 induced cultures restimulated with F13. In contrast, F13-induced cultures showed no signiflcant proliferation in response to F14, and F7 while in response to D122 the viable cell number actually decreased. These results indicate that F13-induced T cells preferentially proliferate in response to F13 and are only minimally cross-stimulated to proliferate by other HER-2 peptides.

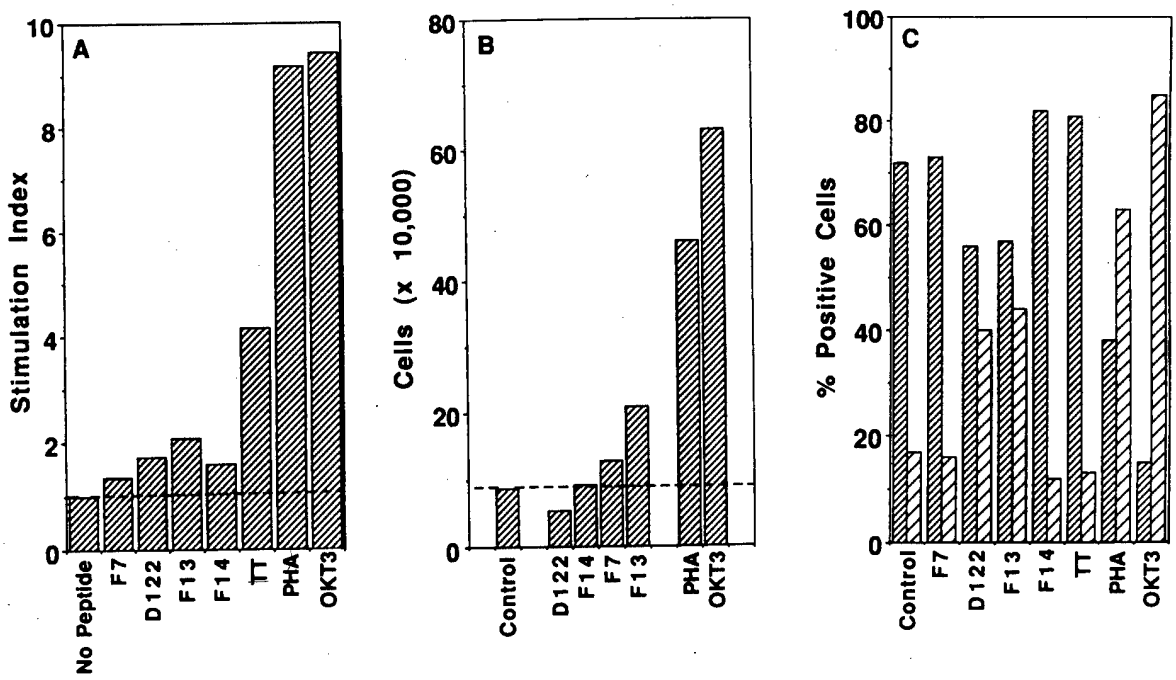

Figure 4. *(A) Proliferative responses to HER-2 peptides F7, F13, F14, and D122 by T-cell cultures induced with the corresponding peptide and expanded in IL-2. (B) Increase in cell number of F13 induced T cells described in (A) after restimulation with either no peptides (control), each of the peptides D122, F14, F7 and F13; or PHA and OKT3 mAb. Experimental conditions were as described in the Materials and Methods. (C) Cell surface phenotypes of peptide-induced cultures described in (A), after restimulation with each peptide and expansion in IL-2* ▨ *$CD4^+$ cells,* ▨ *$CD8^+$ cells.*

To define the T cell phenotype, the resulting PBMC cultures, were analyzed for CD4 and CD8 antigen expression. Analysis of the phenotype of the control cultures was performed in parallel. The results are shown in the Figure 4C. Most cells in the primary stimulated cultures were $CD4^+$ T cells, however there were some significant differences between cultures. F7- and F14-stimulated cultures contained 12 and 16% $CD8^+$ cells respectively, while D122 and F13-stimulated cultures had a significantly higher proportion of $CD8^+$ cells (40 - 44%). Control cultures which were stimulated only with autologous PBMC and IL-2 were of predominantly of the CD4 phenotype while in PHA and OKT3 stimulated cultures $CD8^+$ cells were in majority. These results indicate that, for the same donor, stimulation with each peptide affected in a different way the proliferation of either $CD4^+$ or $CD8^+$ cells or both. This does not reflect the ability of one or another subset to proliferate better in the presence of IL-2. Control cultures which were not stimulated with exogenously added peptides, and TT-stimulated cultures showed a predominantly $CD4^+$ phenotype. Inhibition studies using anti-HLA-mAb indicated that HER-2 peptides induced proliferation was inhibited by anti-MHC class II and at lesser extent by anti-MHC-class I Abs suggesting that the responder cells are T cells (data not shown).

Each set of HER-2 stimulated lymphocytes showed different proportions of $CD4^+$ and $CD8^+$ cells. Since this may reflect the ability of each peptide to induce cytokines, which can affect the proliferation of each T cell subset, the capacity of each HER-2 induced T-cell line to secrete IFN-γ and IL- 10 was determined. These cytokines have been associated with Thl and Th2 types of responses, respectively (19-21). Since cells cultured in IL-2 usually produce background levels of IFN-γ, all peptide-stimulated T-cell lines and control lines were washed and cultured in complete RPMI medium without IL-2 for two days, before being stimulated with OKT3 and PMA. The results of one representative experiment (of two experiments performed) are presented in Figure 5.

T-cell lines stimulated by F7, F13, and F14 produced IFN-γ at higher levels than observed in control cultures stimulated with autologous PBMC but not with peptides. The highest levels were observed with F13 and were similar to the levels induced by TT-induced T cell lines. Interestingly, a different pattern of IL-10 secretion was observed. IL-10 secretion by F13-stimulated T cells was only slightly above the control levels. However, high levels of IL-10 were found in the supernatants from F7- and F14-stimulated T cells. The levels of IL-10 were almost half the level of IL-10 produced by TT-induced T cells. In contrast, while the levels of IFN-γ produced by D122-induced line were higher than those produced by the control cultures, the levels of IL-10 produced by the D122-induced line were minimal. These results show a good correlation between the IL-10 secretion and the $CD4^+/CD8^+$ ratio of T cells from HER-2 peptide induced T

cell lines. F7-, F14- and TTinduced T-cell lines secreted high levels of both IFN-γ and IL-10. Conversely, D122- and F13-stimulated cultures secreted different amounts of IFN-γ but low amounts of IL-10. This may be suggestive of a Thl function for the F13 peptide in this patient and for a Th2 function for the F7 peptide in the same patient.

Discussion

Recognition of HER-2 epitopes by $CD8^+$ cytotoxic T lymphocytes has been extensively documented (2-5). However significantly less information is available about the recognition of HER-2 epitopes by $CD4^+$ cells. Although $CD4^+$ cells may not be always involved in tumor lysis in breast and ovarian cancer, helper T cells may be essential for initiating, sustaining and amplifying an anti-tumor response. CTL induced by stimulation with Ag in the presence of co-stimulation with B7- may become exhausted by the interaction with B7- tumor cells. The presence of Ag specific $CD4^+$ T cells may provide the "self-help" iieeded to sustain CTL responses (22, 23). Thus Thl cells recognizing peptides derived from the processing of HER-2 may produce cytokines (IFN-γ, TNF-α/β) that are thought to provide help for CTL function. The same HER-2 enitones mav produce Th2 cytokines in association with other MHC-class II types.

The objectives of this study were to (a) determine whether HER-2 peptide recognition occurs in healthy donors and in ovarian cancer patients with advanced disease and (b) whether distinct HER-2 peptides differ in their ability to modulate the cytokine secretion potential of the T cells from the same donor. In this report we present evidence that T cells responsive to multiple epitopes on a self-protein, HER-2, exist *in vivo* in healthy donors as well as in ovarian cancer patients. These cells can be stimulated to proliferate and expand *in vitro* and can secrete Th1 and Th2 cytokines. The observed *in vitro* responses of normal T cells to multiple peptides derived from HER-2 cannot be attributed to mitogenic effects by a particular peptide since (a) different peptides elicited PBMC proliferation in different donors and (b) three peptides containing the same P1 anchor for the same MHC-class II molecules failed to induce proliferative responses with the same frequency as four other peptides. Our analysis revealed that PBMC from ovarian cancer patients after chemotherapy responded less frequently than PBMC from healthy donors to the same peptides.

The reasons for the reduced frequency of responses in PBMC from ovarian cancer patients are still unknown. One possibility, to be addressed in future studies, is whether the ability of T cells from patients to respond is affected by chemotherapy. Chemotherapy can eliminate proliferating reacting clones to HER-2 peptides. An alternative is that individuals susceptible to ovarian cancer may be less responsive to self-antigen. This hypothesis could be tested in future studies using HLA-typed ovarian cancer patients. At this moment there is unknown whether there is a

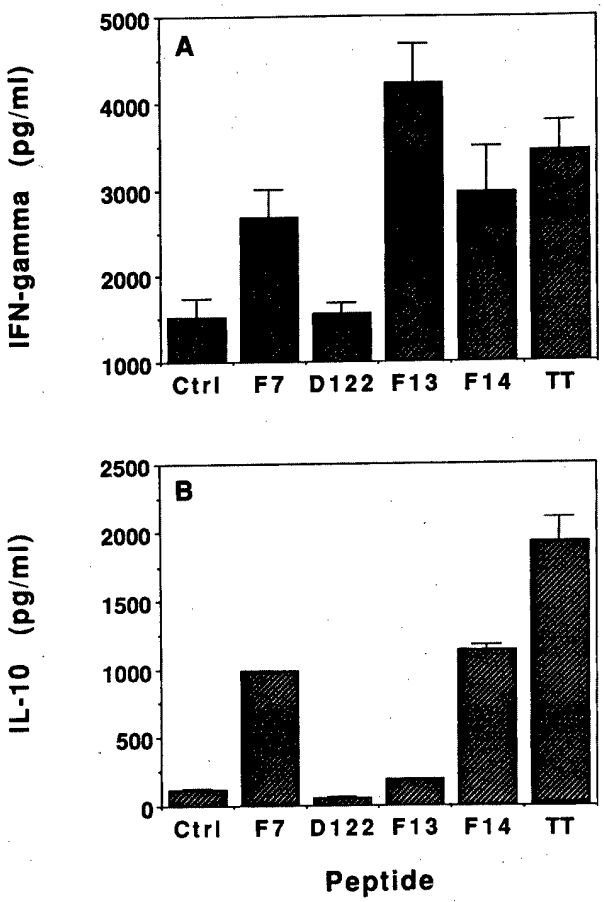

Figure 5. *Cytokine secretion by HER-2 peptides-induced T-cell lines. Equal numbers of cells (10^5) from each culture were plated in 96-well plates and incubated with PMA and OKT3 mAb as described in Materials and Methods. Cytokine expression was determined by ELISA. The concentrations were calculated by comparison with standard plots of IL-10 and IFN-γ in the same assays.*

disproportionate expression of MHC-class II allele in the cancer patients that may account for the different response pattern to the peptide antigens. Other possibilities currently under investigation are whether (a) $CD4^+$ T cells from these patients are anergic to these peptides or (b) their response is suppressed (1, 22). Suppressive effects due to disease progression may account for the lack of responses to these peptides at this stage. Although the group of patients with stable disease is relatively small to allow conclusive comparisions to be made, three of seven patients with stable disease responded to F13 while only two of eighteen patients with progressive disease (11%) responded to F13. In several cases when both PBMC and TAL were available from the same patient, we found that only PBMC responded to these peptides, suggesting the presence in situ of potentially negative regulatory mechanisms. Our preliminary results on the pattern of cytokine responses to F7 and F13 show that for a number of patients IL-10 was detectable at 48h in the

peptide induced cultures but the levels of IFN-γ were below the levels of detection of the assay (10 pg/ml). PHA stimulated PBMC from the same donors secreted both IFN-γ and IL-10. In other patients, only F7induced PBMC secreted TNF-α and/or IFN-γ (Melichar *et al* manuscript in preparation).

In our study, PBMC from ovarian cancer patients responded less frequently to F13 than did PBMC from healthy donors. The frequency of responses to F7 and F13 in healthy donors (54 and 62%, respectively) does not correlate with the frequency of expression of HLA-DR4 (25%) in the human population, suggesting that these peptides can be presented by other class II molecules.

Our current study also sought to elucidate the ability of HER-2-peptide stimulated T cells to expand and secrete cytokines. In this case we studies PBMC from a patient who had shown a stable response to several HER-2 peptides over a six month period. T cell lines of predominantly $CD4^+$ phenotype were readily expanded by restimulation with these peptides and low concentrations of IL-2. Interestingly, the resulting T cell lines differed in their proportions of $CD4^+$ and $CD8^+$ cells in their pattern of IFN-γ and IL- 10 secretion.

One possible explanation for these observed differences is that peptides F13 and F7 function as Thl and Th2 epitopes respectively in association with certain MHC-class II molecules. Both F7 and F13 contain a set of P1-P4-P6 anchors for HLA-DR4, though these sequences differ in charge at the P4 anchor: R (782) in F7 and E (892) in F13 correspond to the motifs for peptide binding sites to the DRBl*0402 and DRBl*0401/0404 alleles (12, 23). Phenotypic analysis of cells in the T cell lines stimulated by these peptides revealed a significantly larger population of $CD8^+$ cells in F13- than in F7- stimulated T cell lines from this donor. Furthermore, a T cell response to the epitope HER-2:783-797 mapped with the peptide SRLLGICLTSTVQ was detected in a breast cancer patient with high level of HER-2 auto-antibodies (1). F7, HER-2 (777-789) overlaps with HER-2:783797 in the area 777 - 783. The possibility that T cells stimulated by F7 can provide help for Ig synthesis deserves further consideration.

The fact that T cells from healthy donors and ovarian cancer patients respond to HER-2 peptides, indicates that tolerance to some of these self-epitope is not induced. Ongoing studies aim to determine whether T cells induced by these peptides recognize the HER-2 protein, the restriction element and the dominant epitopes for induction of a Th1 response. The implications of the observed responses in immunity to, or progression of ovarian cancer deserve further consideration as to whether the responses to these peptides correlate with HER-2 expression, stage and clinical outcome. Such studies are currently in progress in our laboratory. The results presented in this study should be useful for investigation of the mechanisms of Ag specific immunity, auto immunity, tolerance and design of epitope specific tumor vaccines.

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Proliferative and Cytokine Responses to HER-2 Peptides in Breast Cancer Patients

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Running title: Proliferative responses to HER-2 peptides

Abbreviations used in this paper: Cytotoxic T lymphocytes, CTL; Peripheral blood mononuclear cells, PBMC; Antigen, Ag; Polymerase chain reaction, PCR; T cell receptor, TCR; HER-2/neu proto-oncogene, HER-2; stimulation index, S.I.; standard deviation, SD; tetanus toxoid, TT; influenza hemaglutinin, HA; wild-type, w.t.; HER-2 intracellular domain, ICD; HER-2 extracellular domain, ECD.

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ABSTRACT

Previous studies have characterized the reactivity of $CD8^+$ CTL with ovarian and breast cancer. There is little information about the antigens and epitopes recognized by $CD4^+$ T cells in these patients. In this study we analyzed the ability of T cells from PBMC of breast cancer patients to recognize HER-2/neu (HER-2) peptides. We found that 13/18 patients responded by proliferation to at least one of the HER-2 peptides tested. Of these peptides, one designated G89 (HER-2:777-789) was recognized by T cells from 10 patients. 7/9 responding patients were $HLA\text{-}DR4^+$, suggesting that this peptide is recognized preferentially in association with HLA-DR4. Analysis of the specificity and restriction of the cytokine responses by G89-stimulated T cells revealed that these cells secreted significantly higher levels of IFN-γ than IL-4 and IL-10 suggesting priming for a Th0-T helper 1 (Th1) response. The same pattern of responses was observed to the intracellular domain (ICD) of HER-2 suggesting that G89-stimulated T cells recognized epitopes of the HER-2 protein in association with HLA-DR4. Since HLA-DR4 is present in 25% of the humans, characterization of MHC-class II restricted epitopes inducing Th1 responses may provide a basis for the development of multivalent HER-2 based vaccines against breast and ovarian cancer.

INTRODUCTION

Studies in animal models have demonstrated a significant role for T lymphocytes in anti-tumor immunity and have shown that $CD8^+$ and $CD4^+$ cells can mediate tumor rejection (1). Furthermore, trials in melanoma with tumor-reactive $CD4^+$ and $CD8^+$ T cells have shown clinical responses (2). Therefore, in recent years significant emphasis has been placed on identifying epitopes recognized by tumor reactive $CD8^+$ CTL. A remarkable feature of these Ag is that they are non-mutated self-proteins (3). This raises the possibility that $CD4^+$ cells recognizing epitopes on the same self proteins in the context of MHC-I and MHC-II may be also present in cancer patients (4). $CD4^+$ cells may express either direct killing or play a regulatory role for the differentiation of other $CD4^+$ cells and of tumor reactive CTL (5).

The known repertoire of tumor Ag recognized by $CD4^+$ cells is limited. There is little information on the restriction elements operating in each Ag system and the nature of responses (Th1/Th2) induced by self-peptides activating $CD4^+$ cells. Topalian et al (6, 7) have identified HLA-DR4.1 (HLA-DRB1*0401)-restricted tyrosinase peptides, that stimulated Th1 cytokine secretion by $CD4^+$ melanoma TIL. The ability of tyrosinase peptides to mount a Th1 response was shown to be dependent on the binding affinity of the peptide to HLA-DR (7). Yoshino et al have shown that $CD4^+$ TIL secrete Th1 cytokines when presented with heat shock proteins (hsp) associated with HLA-DR suggesting that they may recognize peptides complexed to such proteins (8). Cytotoxic $CD4^+$ CTL were shown to recognize a shared HLA-DR15 melanoma associated Ag (9). MHC-class II-restricted Th1 cytokine secretion by long-term cultured $CD4^+$ TIL has also been reported in breast cancer patients, suggesting a HLA-DR4

associated response (10). Finally, autologous tumor specific $CD4^+$ CTL have been also demonstrated in sarcoma restricted by HLA-DR4 and HLA-DR15 (11).

Together, these studies have shown that *in vitro* cultured $CD4^+$ cells of TIL can recognize class II associated Ag. There is little information on the ability of class II associated peptides from these tumor Ag to induce and restimulate a response in healthy donors and patients with cancer. Proliferative responses to PBMC reflecting responses by T cells to mutated ras protein and peptides carrying the same mutation have also been detected in pancreatic and colon cancer patients vaccinated with the corresponding peptide (12-13). In contrast, responses to wild-type (w.t.) or mutated ras have not been found in healthy donors (12). A number of studies suggest that the immunity directed against mutated tumor proteins may be targeted to w.t. epitopes (14, 15). Recent studies show that breast cancer patients developed anti-p53 antibodies and T cells that proliferated in vitro in response to w.t. p53 only when mutated p53 accumulated in their tumors (15), suggesting that the enhanced presentation of w.t p53 was leading to a Th2 response. Similarly, a breast cancer patient with HER-2 overexpression ($HER\text{-}2^{hi}$) and anti-HER-2 Ab developed T cells that proliferated in response to both HER-2 protein and short synthetic peptide (16).

Although these results suggest *in vivo* priming by enhanced presentation of self-peptides due to either overexpression or to shorter protein half-life there is little information on the ability of $CD4^+$ cells from healthy donors and from cancer patients who do not overexpress HER-2 ($HER\text{-}2^{lo}$)to respond to HER-2 peptides. This is important because

identification of $CD4^+$ cells reacting with self-peptides may allow not only identification of "protective" tumor Ag, but allow optimization of design of tumor vaccines, by incorporating "self-helper" peptide(s) that can amplify and spread a Th1 response when the disease progresses.

We recently found that healthy donors responded with higher frequency than ovarian cancer patients to a number of HER-2 peptides. We hypothesized that $CD4^+$ T cells recognizing HER-2 are not deleted from the immune repertoire of healthy individuals (17). Since the patients in that study were not HLA-typed and had advanced disease, we decided to investigate the ability of HER-2 peptides to induce proliferative responses in patients with primary breast cancer of defined MHC-class II type. We used four HER-2 peptides which induced the most frequent responses in our previous studies. We found that 13/18 patients tested (72%) responded to at least one peptide. Peptide G89 induced responses with higher frequency (10/18, 56%) in this group and significantly higher in the HLA-$DR4^+$ patients (7/9, 78%) than the other peptides tested. There was no difference in the pattern of cytokine responses between one patient that overexpressed HER-2 ($HER\text{-}2^{hi}$), and one donor that did not overexpress HER-2 ($HER\text{-}2^{hi}$) suggesting that the ability of patients with localized breast cancer to respond to G89 it is not affected by HER-2 overexpression.

MATERIALS AND METHODS

Subjects. Peripheral blood mononuclear cells (PBMC) were obtained from 18 breast cancer patients and 6 healthy volunteers (three $DR4^+$ and three $DR4^-$). All patients, with one exception, were clinically free of tumor

at the time of study. Of the healthy volunteers three were HLA-DR4$^+$, while the others were DR4$^-$ (i.e. donor 4, DR7, 11, DQ 2, 6; donor 5, DR13, 14, donor 6, DR11, 15, DQ6, 7). Eleven patients had pathology stage I disease, five had stage II, and one had stage 3. One patient (No. 16) had no primary tumor yet was classified as having breast cancer becuase of the presence of a positive lymph node. HER-2 staining for the autologous breast tumors was performed by immunocytochemistry. The tumor size, nodal status, and results of HER-2 tumor staining, are shown in **Table I.**

HLA class II molecular oligotyping. Genomic DNA was extracted from PBMC as described (18-22); This DNA served as the substrate for amplification of a polymorphic locus-specific fragment of the HLA class II gene by polymerase chain reaction (PCR). For the *-DQB1* and *-DRB* loci, the flanking primers used were:

DRB-AMP-A: 5'CCCCACAGCACGTTTCTTG;

DRB-AMP-B: 5'CCGCTGCACTGTGAAGCTCT;

DQB-AMP-A: 5'CATGTGCTACTTCACCAACGG; and

DQB-AMP-B: 5'CTGGTAGTTGTGTCTGCACAC.

Because of the large number of *HLA-DRB* alleles and the numerous shared sequences between different alleles, *HLA-DRB* typing was carried out in a stepwise manner. First, group-specific *HLA-DR* typing was performed using the primers DR-AMP-A and DR-AMP-B. Oligonucleotide typing of this PCR-amplified DNA allowed discrimination of *HLA-DR1, -DR2, -DR3/6,-DR4, -DR5 (-DRw11), -DR7, -DR8/12, -DR9, -DR10, -DR52a, -DR52b/c,* and *-Drw53*. Since there are numerous variants of *HLA-DR1, -DR2, -DR4, -DR5 (-DRw11), -DR6, -DR8/12* and *-DR52b/c,* further discrimination of these subtypes required a second PCR using group-

specific primers plus DRB-AMP-B. They include DRB-AMP-1 for the *HLA-DR1* group, DRB1-AMP-2 or DRB5-AMP-2 for the *HLA-DR2* group, DRB-AMP-3 for the *HLA-DR3, -DR5, -DR6, -DR8, -DR12* group, DRB-AMP-4 for the *HLA-DR4* group, and DRB-AMP-52 for the *HLA-DRB3* genes of the *HLA-DRw52* group. The sequences of the primers were as follows:

DRB-AMP-1; 5'TTCTTGTGGCAGCTTAAGTT;

DRB1-AMP-2; 5'TTCCTGTGGCAGCCTAAGAGG;

DRB5-AMP-2; 5'CACGTTTCTTGCAGCAGGA; and

DRB-AMP-4; 5'GTTTCTTGGAGCAGGTTAAC;

HLA-DRw52-associated *-DRB1* genes (*HLA-DR3, -DR5, -DR6, -DR8*, and *-DR12*): DRB-AMP-3; 5'CACGTTTCTTGGAGTACTCTAC; *HLA-DRw52*; DRB-AMP-52; 5'CCCAGCACGTTTCTTGGAGCT

PCR products were analyzed by electrophoresis. The amplified DNA was blotted to Hybond N+ membranes (Amersham, Arlington Heights IL) hybridized with γ^{32}P-ATP-labeled allele-sequence specific oligonucleotide probes (SSO). *HLA-DQB1* alleles were determined by hybridization with probes corresponding to variable sequences around positions 23, 26, 37, 45, 49, 57 and 70 of the *HLA-DQB1* outermost domain. "Broad" *HLA-DR* groups [*HLA-DR1, -DR2, -DR3/6, -DR4, -DR5 (11), -DR12, -DR7, -DR8, -DR9, -DR10, -DRB3*0101, -DRB3*0201-*0301, -DRB4*0101 (-DR53)*] were determined by hybridization with oligonucleotide probes corresponding to variable sequences around positions 10, 28 and 37 of the *HLA-DRB1* outermost domain. Subtypes of *HLA-DR1, -DR2, -DR3/5/6/8/12, -DR4* and *-DRw52* were determined by hybridization of the respective group-amplified DNA to oligonucleotides corresponding to variable sequences around positions 28, 37, 57, 70 and 86 of the *HLA-DRB1* outermost domain.

HER-2 peptide selection. Peptides tested were selected if they contained the T cell sites in HER-2 predicted by the computer program ANT.FIND.M, the general binding motif for human class MHC-II antigens, and the anchors for a number of MHC-class II antigens: HLA-DR1, -DR3, -DR4, -DR11, and -DQ7 (23-28), the sum of whose allelic frequencies covers between 75 - 100% of the Americans. The general peptide binding motif for various human MHC-class II molecules consists of a position 1 (P1) anchor, i.e. an aromatic or large aliphatic residue in the first 3 - 5 amino acids close to the N-terminus and other major but less essential anchors at P4, P5, P7, and P9 counting from the P1 anchor (26-28). Since many peptides are capable of binding to many different MHC-class II molecules, because their sequences contain overlapping binding motifs for MHC-class II molecules (27, 28), each of the peptides synthesized contained at least two of three anchors for each HLA-DR antigen, and the main P1 anchors for most class II alleles **(Table II)**. In peptides G88, G89, and G90, positions P3 and P4 are occupied by hydrophobic, aromatic followed by aliphatic residues in that order to facilitate peptide binding in different frames.

The binding motifs of synthetic peptides may differ from those of natural ligands because the latter incorporate processing constraints in addition to binding requirements. Thus peptides were synthesized by following, when possible, the common motifs for all MHC-class II molecules defined by pool sequencing of naturally processed peptides (25). In F7, F13 and F14, the sequence was extended to include Pro (P) N-terminal to either the Tyr (Y), (the P1 anchor for HLA-DR1, DR3, DR4, and DQ7), or Trp (W) (reportedly by the P1 anchor for HLA-DR4 and DR11). For comparative studies of the responses associated with HLA-DR4, 13-mer

analogs of F12, F7 and F13 G88, G89 and G90 respectively were selected, using the anchor alignment matching the standard HLA-DR4/DR1 helper epitope, influenza HA peptide (HA:307-319). The predicted binding affinities of these peptides for HLA-DR4 (as IC50) according to Rothbard's algorithm were as follows: HA, 35 nM, G88, 180 nM, G89, 987 nM, and G90, 219 nM (29). Peptides 13-16 residues long **(Table II)** were prepared by the Synthetic Antigen Laboratory of M.D. Anderson Cancer Center, using a solid phase method as previously described (17, 30). Their identity was determined by amino acid analysis. Their purity was 93 - 97% as determined by HPLC. Peptides were dissolved in PBS, aliquoted at 2 mg/ml and stored frozen at -20°C until use. The codes used to identify these HER-2 peptides in this paper were assigned by the Synthetic Antigen Laboratory.

Recombinant intracellular HER-2 domain. The HER-2/neu intracellular domain (ICD: K676-V1255) was cloned by PCR from c-erbB-2 cDNA (provided by Dr. Jacalyn Pierce, NCI). The ICD was expressed in E.coli using a pET vector with an amino terminal His tag. The expression of ICD protein was induced with IPTG and E.coli pellets harvested after an additional 4 hours. Recombinant ICD was purified from inclusion bodies by a combination of Ni^{++} affinity chromatography, size exclusion and ion exchange chromatography. The purified ICD was greater than 95% pure as judged by PAGE and Western analyses (data not shown).

Stimulation and propagation of T cells. Freshly harvested PBMC from breast cancer patients and healthy volunteers were isolated by Ficoll/Hypaque centrifugation. $CD4^+$ and $CD8^+$ T cells were isolated from the plastic using non-adherent fractions. Cells were cultured at 1×10^6

cells/ml in RPMI-1640 (Gibco, Grand Island, NY) medium with 10% pooled human AB serum and antibiotics in 2 ml in each well of a 24-well plate (complete RPMI medium). HER-2 peptides were added to selected wells at a final concentration of 25 µg/ml. In other wells, PBMC were stimulated with 25 ug/ml HA peptide, PHA at a final concentration of 1:100 or medium alone. After 6 days of stimulation with each peptide, cultures were expanded with IL-2 (Cetus) at 20 U/ml for the following week (17, 31). To induce antigen specific T cells, the cells were then "rested" for 3 - 4 days by culture in the absence of IL-2. Then, the cells were stimulated at a 1:1 (stimulator:responder ratio) with irradiated (10.000 Rad) PBMC and pulsed with individual peptides for at least 90 min at 37°C before addition to the cultures as described (17). Control cultures were stimulated with the same number of PBMC in the absence of peptides. For expansion, four to five days later 20 U/ml IL-2 was added to the cultures for seven additional days. Surface antigen expression was determined by FACS analysis using a FACSscan (Beckton-Dickinson, Sunnyvale, CA) with a log amplifier. CD3, CD4 and CD8 antigen expression on T cell cultures was determined by immunofluorescence with corresponding mAb FITC-conjugated (Beckton Dickinson).

Proliferation assays. For proliferation assays 100 µl aliquots were removed from each well of the 24-well plate of primary cultures after 4-6 days as described (16). Quadruplicate samples were cultured in a 96 well plate with 1 µCi [^{3}H]-Tdr in a final volume of 200 µl. The cells were harvested 16 h later, and the radioactivity counted in a Beckman LS3501 liquid scintillation counter (16). A positive proliferative response was

defined as positive when differences in cpm values between cultures that received peptides compared with cultures which did not receive peptides were significant by the unpaired Student's t-test ($p < 0.05$).

Cytokine production. The ability of PBMC to secrete antigen-induced IFN-γ, IL–4, and IL-10 was determined by culturing the PBMC with the corresponding peptides. Supernatants were collected at different times and stored frozen at -20°C. The cytokine concentrations were measured by double sandwitch-ELISA using the corresponding kits provided by BioSource International (Camariyo, CA). The cytokine assays were calibrated with human recombinant IFN-γ, IL-4, and IL-10 to detect each cytokine in the range of 15-1000 pg/ml. The following homozygous B cell lines were obtained from the American Society for Histocompatibility and Immunogenetics Repository (Baltimore, MD) and used as APC for cytokine secretion: E418 (DRB1*1502, DRB5*0102, DQA1*0102, DQA1*0103, DRB1*0601) and WT51 (DRB1*0401, DRB4*0101, DQA1*0301, DQB1*0302).

Statistical Methods. Differences in proliferative responses were analyzed using Student't t-test for unpaired samples. Differences in frequency for class II alleles were assessed using the Cochran Q test (32).

RESULTS

Recognition of HER2 peptides by breast cancer patients. PBMC of breast cancer patients were cultured with HER-2 peptides, or medium alone for 4-6 days. To ensure that any lack of responsiveness of PBMC to HER-2 peptides did not reflect a generalized suppression of responses to antigen or mitogen, all subjects' lymphocytes were tested for their ability to respond to PHA. Since responses to F7 and F13 were previously observed with higher frequency in healthy individuals we wanted to address the question of the ability of the T cells from breast cancer patients to respond to HER-2 peptides, in association with certain MHC-class II types, expression of HER-2 in their primary tumor, and the lymph node status. Since the patients were tested in the order they presented and not based on their HER-2 expression, to increase the sensitivity of detection, responses were considered positive when the cpm in each of the quadruplicate cultures stimulated with peptides were higher than each of the quadruplicate control cultures, on two consecutive days (4 and 5) or (5 and 6). This approach allows to identify responses without arbitrary cut-offs, using S.I.

Emphasis was given to HLA-DR4$^+$ patients because of the recently reported association of HLA-DR and HLA-DR4 with favorable prognosis in breast cancer (33, 34). Since both F7 and F13 contain HLA-DR4 anchors but differ in the length and position of the anchor motifs, we synthesized two 13-mers designated G89 and G90 respectively. Each contained hydrophobic aromatic followed by a hydrophobic aliphatic residue at P3 and P4 (**Table II**). A control peptide based on the sequence of F12 (G88) was prepared (**Table II**) because it had the same pattern of residues in P3 and P4. The responses to these peptides for eight of the 18 patients

tested (including responders and nonresponders) are shown in **Figure 1.** The other three HLA-DR4$^-$ donors responded preferentially to F13 with S.I. of 1.9, 1.7 and 1.5 respectively but no significant proliferation to G89 was observed (data not shown). The ability of PBMC of HLA-DR4$^+$ healthy volunteers to recognize HER-2 peptides was also tested. Significant proliferative responses by the same criteria were detected in three of three HLA-DR4$^+$ individuals after primary stimulation of PBMC with various HER-2 peptides (**Figure 2**). Thus, the ability to recognize sequences of the HER-2 protein is within the realm of the TCR of healthy volunteers as we reported (17).

CD4$^+$ cells responded to G89 (**Figure 2C**). Proliferative responses to HER-2 peptides from all 18 patients tested are summarized in **Table III.** G89 and F7 were recognized by PBMC from ten and eight patients respectively. PBMCs from six patients recognized both G89 and F7. Responses to G90 and F13 were observed in six and three patients respectively. The results show a higher frequency of responses for G89 and G90 containing MHC-class II anchors in P3 and P4 than for their analogs (F7, F13) with the anchors shifted. The frequency of responses to G89 was significantly higher than to other peptides ($p = 0.02$). The results also show preferential association (7/9) of the responses to G89 with the presence of HLA-DR4 ($p = 0.01$). Of the other alleles that were represented, 4 of 5 HLA-DR3 patients responded to G89 but 3 of 4 responders were also HLA-DR4$^+$. 4 of 6 HLA-DR2$^+$ patients responded to G89 but 3 of 4 responders were also HLA-DR4$^+$.

With one exception, all of these patients were free of disease at the time of the experiments. HER-2 was overexpressed only in tumors from

patients 6 ($DR4^-$), 13 and 15 ($DR4^+$). The disease status, tumor size, lymph node status, and tumor grade shown in **Table I** were also compared with the response to these peptides. There was no correlation between proliferative responses and the LN status.

No apparent correlation was found between HER-2 overexpression and proliferative responses to HER-2 peptides. Of the three $HER\text{-}2^{hi}$ patients (two $DR4^+$ and one $DR4^-$) responses to G89 were seen only in patient 13 ($DR4^+$). These responses were stable in both day 4 and 5 of testing. In contrast, patient 15 responded only to F13 (on day 6) (**Figure 1E**) while patient 6 ($DR4^-$) responded only to G88 and F7. Of the seven $HER\text{-}2^{lo}$ patients, five showed responses to G89 on at least two consecutive days of assay, one showed a response on day 5 (S.I. >2.5) (**Figure 1D**), and one failed to respond. This group was too small for statistical analysis to address possible differences in response due to HER-2 overexpression and disease progression. Further analysis focussed on characterizing the cytokine response to G89 by T cells from patient 13 ($HER\text{-}2^{hi}$) and donor 3 ($HER\text{-}2^{lo}$).

To address the specificity of responses, G89 primed PBMC from patient 13 (**Figure 2**) were expanded in culture with IL-2 and re-tested for their proliferative responses to G88, G89, and G90. Freshly isolated monocytes/macrophages were not available from this patient for restimulation and follow-up studies. We used PHA blasts from the same patient as APC. Results in **Figure 3A** show that G89 stimulated cultures (G89R) recognized G89 significantly better than G88 and G90 but the overall level of response was low. In fact no clear improvement in proliferative activity was seen after expansion in IL-2 without

restimulation with peptide. The specificity of responses was characterized in parallel with the response from donor 3 (also HLA-DR4$^+$) because autologous APC were available. Cultured PBMC of this donor (desginated G89L) showed significantly higher proliferative responses to G89 than to G88 at restimulation when presented on autologous adherent fraction of PBMC **(Figure 3B)**. In contrast, the corresponding G90L cells showed significantly lower proliferation to recall with G90, suggesting that G90 may prime the T cells for a cross-reactive epitope.

Secretion of IFN-γ by G89-stimulated T cell lines. Since the levels of IFN-γ and IL-4 in primary cultures were very low or undetectable (data not shown), studies were conducted on secondary cultures. Recent studies have shown that IL-2 is required for Th2 differentiation and IL-4 production (35). To determine the type of cytokine responses to G89, cultures were established in low-dose IL-2 after initial stimulation of PBMCs with G89 from Patient 13 (G89R), and Donor 3 (G89L) and with G90 from Donor 3 (G90L). The ability of these cells to secrete IFN-γ, IL-4, and IL-10 in response to a specific peptide was tested. In preliminary experiments we observed that the levels of IL-4 and IL-10 were low or undetectable. Since secretion of IL-4 and IL-10 may be delayed or HER-2 peptides maybe less efficient in inducing Th2 cytokines we determined that cytokine profile at both 40 and 140 h. The G89R T cells secreted high levels of IFN-γ in response to G89 but not in response to control G88 peptide (**Figure 4**). These cells also secreted significantly less IL-4 and IL-10 than IFN-γ after 40 and 140 h in culture, suggesting a preferential Th1 or Th0-Th1 response to G89. A similar pattern of specific cytokine secretion was observed with the G89L T cells. However since the reponses

determined were obtained with short-term bulk cultures, and background levels of IL-4 and IL-10 were present, we define this reactivity as Th0-Th1. The G90L T cells showed significant cross-reactivity with G88 and secreted significantly more of IL-10 than did G89-induced T cells. Although the levels of IFN-γ were higher than the levels of IL-4, the levels of IL-10 were higher than the levels of IFN-γ. Although it may be possible that earlier levels of IFN-γ secreted in response to G90 may have been higher, the high levels of IL-10 may suggest the presence of non-specific Th2 cells activated following the initial G90 stimulation.

To address whether IFN-γ was secreted in response to G89 and of the ICD (which contains this peptide), in association with HLA-DR4, G89R- and G89L- T cells were tested for cytokine secretion in response to G89 presented by PBMC of different phenotypes (**Table IV**). Significantly higher levels of IFN-γ than IL-4 were secreted in response to G89 and ICD when G89 was presented by autologous APC to G89L. A similar pattern of response was observed for the G89R cells. APC (from Donor 3) and responders shared only HLA-DR4 and HLA-DQ6. In the presence of APC that shared *(a)* DR15 and DQ6 with G89L, or *(b)* DQ6 with G89L and G89R, significant levels of IFN-γ were observed in response to G89 but not to ICD. The results also show that the restriction element used by G89L and G89R for recognition of exogenously added peptides is not exclusively HLA-DR4. IFN-γ and IL-4 release was observed in response to G89 (but not to ICD) in the presence of APC sharing HLA-DR15 or HLA-DQ6 with the responders. The same levels of cytokines were seen in response to APC not sharing HLA-DR with the responders. Although the levels of cytokines secreted were much lower than in response to HLA-DR4, a certain level of

"promiscous" recognition was present, consisting always of higher levels of IFN-γ than IL-4. This suggests that although MHC class molecules of DR4- APC could present exogenously loaded G89 in a recognizable form to G89L and G89R TCR, the naturally processed and presented fragment of the ICD may have been derived from the one presented by DR4. A similar pattern of responses, although reduced, has been seen using lymphoblastoid cell lines WT51 (homozygous for DR4) and E4181324 (homozygous for DR15) as APC (data not shown).

DISCUSSION

In this report, we present evidence that PBMC from primary breast cancer patients respond by proliferation in vitro to a number of HER-2 peptides. The responding population consists of $CD4^+$ cells, as shown in this and a previous study (16) and as suggested by the ability of the responding cells to secrete IFN-γ in response to these peptides when presented by MHC-class II. However, given the length of these peptides, the stimulatory potential for $CD8^+$ cells after binding of G89 to certain HLA-class I allele products deserves further investigation. Low levels of proliferation were observed with isolated $CD8^+$ cells in a healthy donor. The frequency of the responses was higher for G89 (56%) than for the other peptides tested, suggesting that G89 may represent an immunodominant epitope in the group analyzed. Of interest, the responses to G89 appeared to associate more frequently with the presence of HLA-DR4 (in 7/9 cases) suggesting that HLA-DR4 may be the presenting element.

Since F7 and G89 are equal in length but differ by one residue at their N- and C-terminal ends, this suggests that the epitope formed by G89 *in vitro* when used at a concentration of ~10 μM is specifically recognized. The frequency of responses appeared not to be related to the binding affinity of these peptides to DR4. The predicted binding affinity of G89 to HLA-DR4 was significantly lower than that of peptides G88 and G89 of the same length.

It is unknown at this time whether for G89, binding to HLA-DR depends on sequence-specific parameters, or is restricted to certain DR4 subtypes or can be presented by different DR molecules. Depending of which P1 frame is used Tyr or Trp can serve as anchors for DRB1*0401 but not for *0404 and *0402. Similarily at P4 negatively charged residues E, D, are accepted by DRB1*0401 and *0404 but not by *0402 allele which accepts positively charged residues such as K/R (36). This suggests that G89 (as well as G90) may preferentially bind to different DR4 subtypes, and use alternative binding frames (i.e. with V, L, M for the P1 frame). Additional studies are required to address the question whether the antigenicity of G89 is associated with the predicted poor binding in a similar fashion as reported for most tumor peptides from self-Ag recognized by human CTL.

T-cell cultures primed with G89 responded at restimulation by secreting more IFN-γ than IL-4 and IL-10, suggesting the preferential activation of a Th1 response. Since the experiments were performed with bulk cultures and not with clones, and IL-4 and IL-10 were detectable, we would rather define this reactivity as Th0-Th1. This response was apparently not directed to a cryptic HER-2 epitope since peptide-primed

cells recognized the ICD. Although the results of this study do not rule out the presence of cross-reactive epitopes with G89 in the ICD, it should be mentioned that a T cell line induced by stimulation with G90 (located in the ICD, at amino acids 886-898) showed cross-reactive recognition with the ECD epitope G88 (450-462). The IFN-γ response to ICD of G89-primed T cells suggests that HLA-DR4 may be the presenting element for a naturally processed epitope similar in structure to G89.

Recent studies to examine proliferative responses in breast and ovarian cancer using HER-2 peptides of various length and randomly selected patients which have not been HLA-typed showed T cell reponses to several HER-2 peptides (16, 17). One of those defined as p783 (HER-2, 783-797), reportedly, activated responses of T cells to the HER-2 protein, in a breast cancer patient (16). Although the magnitude of G89 induced responses was significantly lower than the one reported for p783, our results indicate a trend of increased proliferation to G89. F7(HER-2:776-788) was also found to induce T cell proliferation in both healthy donors and ovarian cancer patients (17). The data in this study suggests that within the area HER-2: 776-797 nests a dominant HER-2 epitope for $CD4^+$ cells. Since HLA-DR4 is expressed in approximately 25% of humans, this epitope may be an important peptide for activation and regulation of T cell differentiation toward a Th1 response. It may also be beneficial for CTL activation and expansion.

That T cells from both healthy donors and patients whose tumors overexpress HER-2 can respond to G89 argues against the induction of tolerance to this epitope, and/or against auto-immune activation of G89-specific T cells by HER-2 only after protein overexpression. In both this

and the previous study with p783 (16) the proliferative responses were observed early, 4 - 6 days after stimulation. This may argue against a primary response to G89. Primary *in vitro* responses of T cells to some foreign Ag have been shown to require significantly longer time (7 - 9 days) to be detected as significant proliferation (37, 38) although it is unknown whether these findings can be extended to self Ag. The low levels of cytokines at primary stimulation may even argue against a recall response, unless the frequency of G89 specific cells is very low. Additional studies are needed to clarify this point. A possibility that needs to be considered is that epitopes such as G89 may induce *in vivo* a limited number of Th1 cells, which may exert a regulatory function. Preliminary studies in our laboratory show that primary stimulation of T cells from healthy donors with either F7 or F12 or F13 followed by culture in IL-2 leads to preferential expansion of F7-responsive cells. These cells secreted high levels of IFN-γ at secondary and tertiary stimulation with F7. This pattern of responses suggests a determinant spreading effect as described for some cryptic epitopes (39) (Anderson BW et al, manuscript in preparation).

Previous studies of HER-2 focussed primarily on characterizing CTL epitopes (40, 41). The observations in different systems, that human tumors are antigenic though poorly immunogenic emphasize the need for development of approaches to induce and augment an immune response to tumor. Although *in vitro* and *in vivo* models show that induction of tumor specific CTL can be achieved by co-stimulation (42), the observed activation of Th1 response by the same tumor Ag recognized by CTL, suggests an involvement of $CD4^+$ cells in the reaction to tumor. It also raises the question whether the G89-induced Th0-Th1 response plays a

protective role, during tumor spread or if it is down-regulated by Th2 cytokines subsequent to recognition of other peptides after HER-2 overexpression. In this context, the characterization of epitopes that regulate Th1 responses, which can in turn control the spread of Th1/Th2 responses by other self-peptides, may have important implications not only for CTL induction but also for understanding the regulation of human tumor immunity.

LEGENDS TO THE FIGURES

Figure 1. Histograms of selected representative patterns of proliferation for eight breast cancer patients. **(A)** Patient No. 13, average. NP value, 625 cpm. **(B)** Patient No. 7, average NP value 862 cpm, **(C)** Patient No. 2 average NP value 268 cpm, **(D)** Patient No. 9, average NP value 272 cpm; **(E)** Patient No. 15, average NP value 447 cpm, **(F)** Patient No. 12, average NP value 313 cpm, and **(G)** Patient No. 1, average NP value 268 cpm and **(H)** Patient 5, average NP, value 568 cpm. Each determination was performed in tetraplicate; cpm for each of the replicates are represented by one triangle. Patients in panels **A-F** are HLA-DR4$^+$, those in panels **G** and **H** are HLA-DR4$^-$. Responses to G89 were considered positive in panels **A-D** and **F** (HLA-DR4$^+$ patients) and in panel **G** (HLA-DR4$^-$ patient). Freshly isolated PBMC from each donor were stimulated with peptides at a final concentration of 25 µg/ml. Responses were determined on 100 µl aliquots of cells removed from cultures on days 4 - 6 and tested for proliferation. Responses are shown for cultures stimulated for either 4 or 5 days. In most instances significant proliferation was observed on two consecutive days (days 4 and 5 or days 5 and 6).

Figure 2. Proliferative responses to HER-2 peptides by **(A)** PBMC from Patient 13 (designated R) (▲), **(B)** PBMC from healthy donor 2 (▲) determined in the same experiment, and **(C)** CD4$^+$ and CD8$^+$ cells from healthy donor 3 (designated L) (■). Both donors were HLA-DR4$^+$. The MHC-class II phenotypes of the three healthy donors tested were as follows: Donor 1: DR4, 13 (w52), DQ1, 3; Donor 2: DR4, 15 w53, DQ6, 7; and Donor 3: DR4, 13 w52, DQ1, 3. **(A, B)** Responses were determined five days after incubation with peptides in the same experiment for both

donors. Responses in donor 2 were determined only to peptide F7, F13 and F14. Similar results were obtained for donor 3. (C) Responses were determined after 4 days incubation with peptide including the last 16 h the presence of ^{3}H-Tdr. Equal numbers of autologous plastic adherent PBMC were used as APC.

Figure 3. (A). Specificity of proliferative responses of G89R T cells (derived after expansion in IL-2 of primary stimulated PBMC from Patient 13). PHA blasts from the patient 13 were used as autologous APC. **(B).** Specificity of proliferative responses of G89L and G90L (derived from donor 3). The G90L line was developed by priming with G90. Autologous plastic-adherent PBMC were used as APC.

Figure 4. Cytokine secretion by G89R, G89L, and G90L T cells (5 x 10^4 cells each) in response to G89 and G90 respectively pulsed on autologous irradiated PBMC from donor 3 (1 x 10^5). G88 was used as control. Between 70-80% of cells had $CD4^+$ phenotype. Cytokine secretion by G89R, G89L and G90L was measured in the same experiment as described in the Materials and Methods. IFN-γ (▨), IL-4 (▨), IL-10 (▨). *Indicates that levels of this cytokine were below the sensitivity of the assay (2 pg/ml).

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Table I. Characterization of disease status of breast cancer patients

Patient No.	Disease Status	Tumor (cm)	Nodes +	Grade	HER-2 staining (+/-)
1	REC	2.1	0	1	-
2	NED	0.7	0	2	-
3	NED	3	3	2	-
4	NED	3	3	2	-
5	NED	1.2	0	2	-
6	NED	1.2	0	1	+
7	NED	2.5	0	2	-
8	NED	2.5	0	2	-
9	NED	1	0	2	-
10	NED	1.0	0	2	N.D.
11	NED	1.5	0	1	-
12	NED	1.2	0	3	-
13	NED	8	19	2	+
14	NED	1.0	0	-	N.D.
15	NED	1.8	0	1	+
16	NED	N.T.	1	-	-
17	NED	1.5	0	-	N.D.
18	NED	1.5	1	1	N.D.

Patients are listed in alphabetical order. REC = recurrent disease; NED = no evidence of disease.

N.D. = not determined, (the specimen was not in M.D.A), (-) = not obtained, N.T. = no primary tumor, but one lymph node positive.

Table II. HER-2 peptides used in this study

Peptide Code	Position	Sequence
HA	307 – 319	P K *Y* *V* K Q N T L K L A T
F12	449 – 464	G I S *W* *L* G L R S R E L G S G L
G88	450 – 462	I S *W* *L* G L R S R E L G S
F14	474 – 487	T V P *W* D Q L F R N P H Q A
F7	776 – 788	G S P *Y* *V* S R L L G I C L
G89	777 – 789	S P *Y* *V* S R L L G I C L T
F13	884 – 899	V P I K *W* *M* A L E S I L R R R F
G90	886 – 898	I K *W* *M* A L E S I L R R R

HA is the helper influenza HA peptide. The Tyr (Y) and Trp (W) italicized in positions 3 or 4 may constitute P1 anchors. Similarly the Val (V), Leu (L) and Met (M) italicized in positions 4 and 5 respectively may also constitute P1 anchors.

TABLE III. Summary of proliferative responses of breast cancer patients to HER-2 peptides.

PATIENT #	DRB	DRB	DQB	DQB	NP	HA	G90	G88	PHA	F7	F13	G89	F14
1	3	11	301	201	C	-	+	-	+	+	+	+	+
2	4	7	301	201	C	-	-	-	+	+	-	+	-
3	2	4	303	602	C	-	-	-	+	-	-	-	-
4	2	7	201	502	C	-	+	-	+	+	+	+	+
5	6	7	303	303	C	+	+	+	+	+	-	-	+
6	1	7	303	501	C	-	-	-	+	-	-	-	-
7	2	4	302	602	C	+	+	+	+	+	+	+	+
8	3	4	201	201	C	-	-	+	+	-	-	+	-
9	2	4	302	602	C	-	-	-	+	+	-	+	+
10	2	2	602	602	C	-	-	-	+	-	-	-	-
11	3	4	301	501	C	+	-	-	+	+	-	+	-
12	2	4	301	602	C	-	-	-	+	-	-	+	-
13	3	4	302	604	C	+	+	-	+	-	-	+	-
14	3	6	402	501	C	-	-	-	+	-	-	-	-
15	4	7	301	301	C	-	-	-	+	-	-	-	-
16	6	11	603	604	C	-	+	-	+	-	-	-	-
17	6	6	N.D.	N.D.	C	-	-	-	+	-	-	-	-
18	8	8	301	501	C	-	-	-	+	-	-	+	-
TOTAL						**4**	**6**	**3**	**18**	**8**	**3**	**10**	**4**
% Positive						**22.2**	**33.3**	**16.7**	**100**	**44.4**	**16.7**	**55.6**	**22.2**

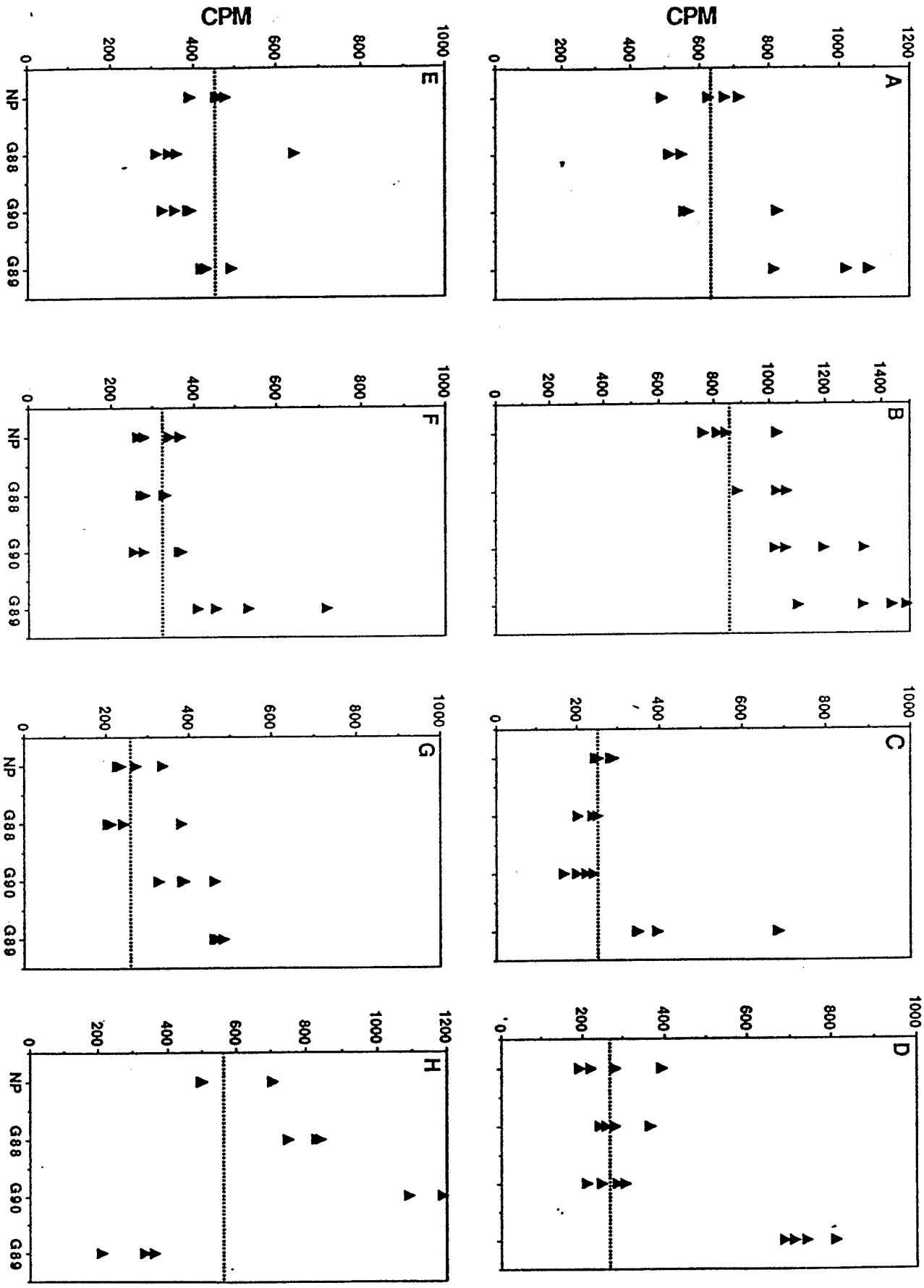
Significant proliferative responses according to Student's t-test were designated (+). Responses not significantly different from those in control were designated (–). All patients tested showed significant proliferation to PHA (data not shown) The allelism of the HLA-DQ has been determined and is listed. Values for control cultures that were not stimulated with peptides (NP) are listed as (C).

Table IV. MHC-class II restriction of peptide and HER-2 protein recognition by G89 induced T cell lines.

			G89L* (DR4,15, DQ6,7)		G89R* (DR4, 3, DQ3,6)	
	APC	Peptide	IFN-γ	IL-4	IFN-γ	IL-4
A.	DR4, 15; DQ6,7	G89	250	10.5	341	39.5
		G88	26	11.1	73	25.0
		ICD	298	17.9	662	10.0
	APC only	G89	<2	<2	<2	<2
B.	DR10, 15, DQ1.6	G89	155	17.8	125	10.4
		G88	<2	<2	<2	<2
		ICD	<2	<2	<2	<2
	APC only	G89	<2	4.7	<2	<2
C.	DR7, 11, DQ2.6	G89	18.2	<2	59	<2
		G88	2.2	2.5	<2	<2
		–	<2	<2	<2	<2

*The G89L and G89R T cell lines were stimulated with 1 μM of HER-2 peptides (G89, G88) or 1 μM of the HER-2 intracellular domain (ICD) in the presence of **(A)** autologus APC, i.e. APC sharing all DR and DQ with G89L and, HLA-DR4 and HLA-DQ6 with G89R; **(B)** APC sharing only DR15 and DQ6 with G89L, and only DQ6 with G89R; and **(C)** APC sharing only HLA-DQ6 with G89L. Supernatants were collected after 40 h in culture. Cytokine levels were determined as described in Materials and Methods.

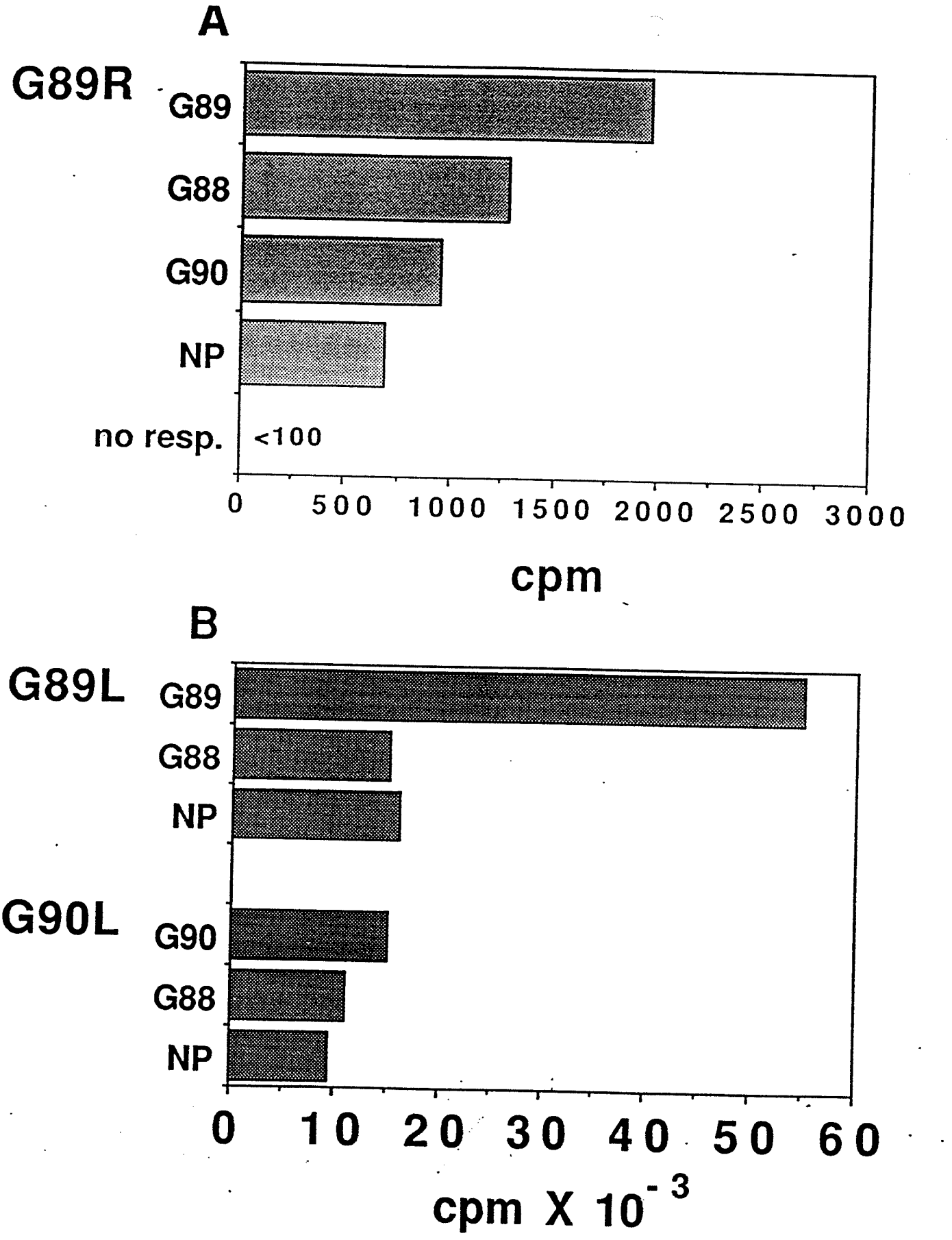
Figure 1



cpm
0
200
400
600
800
1000
1200
1400
1600
A
NP
G90
G88
F7
F13
G89
F14
TT
HA
B
NP
F7
F13
F14
HA
TT
0
1000
2000
3000
4000
C
CD4+
NP
G89
G90
CD8+
NP
G89
G90

Figure 2

Figure 3


A
G89R
G89
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NP
no resp.
<100
0
500
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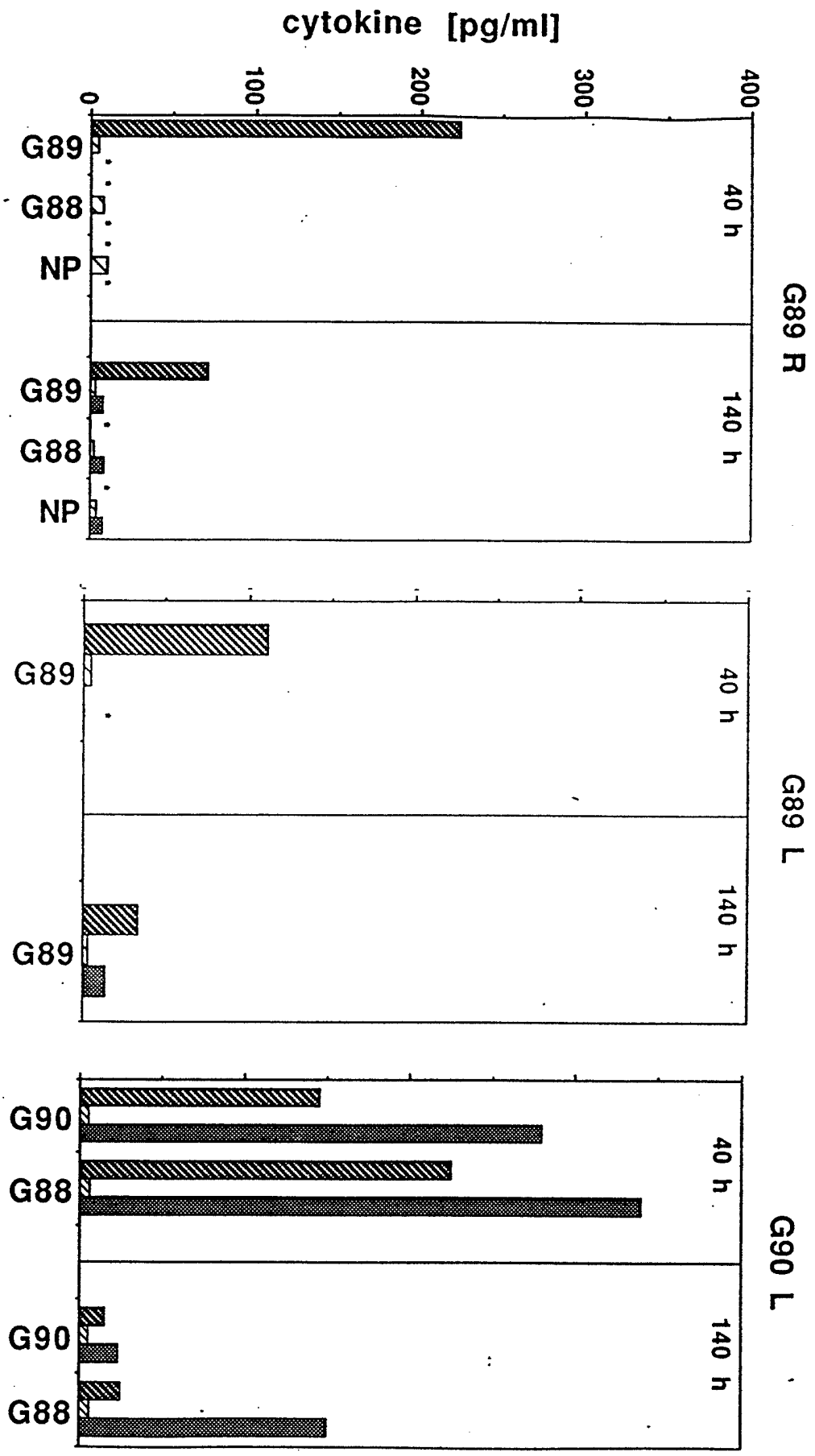
cytokine [pg/ml]
0
100
200
300
400
G89
G88
NP
40 h
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G89 R
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40 h
140 h
G89 L
G90
G88
G90
G88
40 h
140 h
G90 L

Figure 4

Growth and Target Recognition of Tumor Infiltrating Lymphocytes from Human Breast Cancer

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Running Title: Characterization of breast CTL-TIL

Key Words: breast cancer, CTL, tumor antigen

Abbreviations used in this paper: tumor infiltrating/associated lymphocytes, TIL/TAL; Cytotoxic T lymphocytes, CTL; T cell receptor, TCR; HER-2/neu proto-oncogene, HER-2;

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ABSTRACT

In the present study, we isolated tumor infiltrating lymphocytes (TIL) and tumor associated lymphocytes (TAL) from solid tumors and malignant effusions respectively, of 27 breast cancer patients. Significant in vitro proliferation and expansion was observed in 20 of 27 distinct samples. The TIL cultures were initiated using OKT3 mAb in the presence of moderate concentration (25-50 U/ml) of IL-2 followed by 100 U/ml of TNF-α. TAL were not stimulated with OKT3 mAb. Six of six distinct TAL grew in culture as predominately $CD4^+$ lines. In contrast, due to the small sample size, only 14 of 21 (66%) of primary breast TIL expanded in culture, and were predominantly of $CD8^+$ phenotype. Autologous tumor lysis was observed in seven of eight cases tested. Three of the eight TIL/TAL preferentially lysed autologous tumor. In addition to the HER-2 peptide GP2, peptides E75, C85, E89 and E90 were recognized, suggesting that multiple HER-2 epitopes are presented to breast TIL/TAL in the tumor environment. This may be of interest in developing vaccine strategies for therapeutic management of breast cancer.

INTRODUCTION

Breast cancer represents the most common cancer in women and the second most common cause of cancer death in the U.S. (1). Despite the improvements in early diagnosis and treatment over the last 60 years, the mortality from breast cancer remained the same over this period (1), underscoring the need for novel therapeutic approaches toward this disease. Despite the fact that surgery plus chemotherapy remove most of the primary tumor, and the residual tumor may be easier to eliminate by a strong immune system (than e.g. metastatic melanoma), there are few studies on the immune recognition of human breast cancer. Strong evidence for local activation of mononuclear cells in situ, was exemplified by expression of cytokines and cytokine genes (2, 3). Selective expression of early activation markers was found in breast TIL associated with depressed levels of IL-2 and IL-2R, suggesting that TIL may be in a relatively anergic state (3,4). These results are indicative of the presence of antigenic molecules for T cells at the primary tumor site.

There are few studies on the feasibility of propagation of tumor infiltrating lymphocytes (TIL) from primary breast tumors. There is also limited information on the nature of the antigens recognized by breast CTL. This is compounded by difficulties in isolation of breast TIL from small size tumors (<0.5 cm). Evidence that breast TIL can recognize autologous tumor has been recently reported (5-10). Breast TIL propagated in high concentration of IL-2 plus LAK cell supernatant (5, 8) exhibited infrequent preferential lysis of autologous tumor but more frequently exhibited specific Th1 cytokine secretion (5, 9, 10). Reversal of anergy using OKT3 mAb and repeated stimulation with autologous tumor in the presence of moderate concentrations of IL-2 resulted in a high proportion of tumor specific CTL (7, 8). Since the latter is dependent on the availability of autologous tumor, an alternative approach is to use Ag recognized by these TIL to propagate tumor specific CTL. Using different methods, breast TIL were isolated that recognized MUC-1, MAGE-1 and a HER-2 peptide designated GP2 (11-13). The Ag specificities recognized by these CTL may not always be present on the autologous tumor at the time of collection or the specific CTL frequency may be low (in these patients the disease

progresses). However they may reflect existent responses to sensitizing Ag expressed earlier by the tumor. The recent identification of several CTL epitopes on HER-2 (14), presented by HLA-A2 provide a model in which these questions can be addressed.

The objectives of this study were two fold: **(a)** to determine whether breast TIL can be expanded from small primary breast tumors; **(b)** to determine whether breast TIL/TAL can lyse breast tumors and/or recognize HER-2 peptides defining CTL epitopes. Since high dose cytokine may lead to proliferation of different populations, while repeated stimulation with tumor may lead to restricted specificities, we used an alternative approach for T cell propagation consisting of co-culture of breast TIL in the initial presence of autologous tumor (when available) and OKT3 mAb and moderate concentrations of IL-2 (25-50 U/ml). We found that breast TIL from small primary tumors isolated by enzymatic or non-enzymatic digestion can be expanded in vitro. In addition to GP2, HER-2 peptides previously found to be recognized by ovarian TAL were also recognized by breast TAL.

MATERIALS AND METHODS

Isolation and culture of breast TIL and TAL

Fresh tumor tissue was obtained from primary breast tumors in fourteen patients. Tumor infiltrating lymphocytes (TIL) were isolated after enzymatic digestion as described (9) for 4 hours at 37°C. If sufficient number of cells were present (> $2x10^6$ cells), tumor cells and lymphocytes were separated over 75/100% Ficoll gradients. The lymphocyte enriched fraction containing 10-20% tumor cells was cultured in complete RPMI medium with 10% FCS, gentamycin and IL-2 (50 Cetus units/ml) in 24 well plates (Costar, Cambridge, MA). In case of recovery of a small number of cells, cultures were propagated in 48 well plates (Becton-Dickinson, Franklin Lakes, N.J.) in medium with IL-2 at an initial cell density of 10^6 cells/ml. In many instances, breast TIL from primary tumors showed slow growth during the first week in culture. To facilitate their expansion, they were initially cultured on plates coated with OKT3 mAb as described (8) in complete medium supplemented with 100 U/ml TNF-α (15).

When the tumor specimen was small (≤ 0.5 cm) enzymatic digestion, even for a limited period of time, lead to very low recovery of TIL. Since these cells were probably present in very low numbers, and because of their low recovery, they could not be expanded in cultures. We therefore used an alternative approach. This approach is based on the use of small tissue fragments for the growth of TIL (16). The tumor tissue was cut in pieces (<1.0 mm) which were cultured in complete RPMI medium with 25 U IL-2/ml and 100 U/ml of TNF-α for the first 16 hours. Afterwards, OKT3 mAb (10 ng/ml) was added to the cultures. After 2 - 3 days, leukocyte migration out of the tumor pieces was observed. Afterwards, tumor pieces were dispersed by pippetting up and down, the mixture was allowed to settle for 30 - 40 seconds and the cell suspension was replated. This procedure was found to provide better yields of recovery than enzymatic digestion and was used for TIL - 10 through TIL -14.

Malignant effusions were obtained from six patients (pleural effusions in 4 and ascites 2 patients). Isolation of tumor associated

lymphocytes (TAL) was performed as described (17). Briefly, after pelleting, the cells were resuspended in serum free medium and layered over 75/100% Ficoll gradients. Purified lymphocytes were cultured in complete medium without OKT3 mAb. CD8+ cells were isolated on specific mAb coated plates as we described (14).

Phenotype analysis

Expression of T cell populations, of HLA-A2 on TAL/TIL and of HER-2 on tumors were determined using monoclonal antibodies (mAb) BB7.2 (American Type Culture Collection, Rockville, MD.) and Ab-2 specific for the extracellular domain of HER-2 (Oncogene Science, Manhasset, N.Y.). Briefly, the cells were incubated with primary antibody for 30 min at 4°C followed by fluorescence isothiocyanate-conjugated (FITC) goat-anti-mouse IgG (Beckton Dickinson, San Jose, CA.). Expression of CD3, CD4 and CD8 was determined by direct immunoflourescence using OKT3, OKT4 and OKT8 FITC mAb (Ortho Diagnostic, Raritan, N.J.). Surface antigen expression was determined using a FACScan flow-cytometer (Beckton-Dickinson) with a log amplifier.

Synthetic peptides

The following peptides were prepared by the Synthetic Antigen Laboratory at the M.D. Anderson Cancer Center using a solid phase method and purified by HPLC: E75 (HER-2, 369-377) KIFGSLAFL; F53 based on the sequence of GP2 (HER-2, 654-662) IISAVVGIL; E90 (HER-2, 789-797); E89 (HER-2, 851-859) VLVKSPNHV and C85 (HER-2, 971-979) ELVSEFSRM. Identity of peptides was established by amino acid analysis. The purity of peptides was more than 95%.

Cytotoxicity assays

Cytotoxicity was measured in 51Cr release assay as previously described (14, 17). Autologous and allogeneic tumor cells were labeled with ^{51}Cr. TIL/TAL were added at different effector:target (E:T) ratios. To study recognition of HER-2 peptides, T2 cells were incubated with peptides

for 120 min, effectors were added and incubated for 5 h as described (17). Control wells were made with T2 cells incubated with synthetic peptides in the absence of effectors.

RESULTS

Growth and T cell phenotype of TIL and TAL from breast cancer

After isolation, TIL and TAL were propagated with moderate doses of IL-2 (25-50 U Cetus/ml). Lymphocytes from both malignant effusions and solid primary tumors exhibited significant expansion, even TIL obtained from relatively small pieces of tumor (less than 2 cm in diameter). After 1-2 weeks in culture, tumor cells and other non-lymphoid cells disappeared. The culture was continued as necessary to obtain lymphocytes for further studies (usually 3-4 weeks). The doubling time for ten TIL/TAL was three days, for two others four days and for the remaining three, five (2) and seven days (one) respectively. The fold expansion in the first four weeks ranged between 40 and 250 fold (data not shown). Since we could not determine the initial number of T cells in TIL processed without enzymatic digestion we could not establish doubling times. However, the large numbers of T cells present after two weeks in culture suggest a vigorous growth in 4/5 cultures (except TIL-14). Overall, of 14 distinct primary breast tumors processed by enzymatic digestion, nine distinct TIL (64%) were successfully established in culture. These TIL are listed in **Table I.**

A similar pattern of rapid growth was observed for TIL isolated from tumor samples which were not subjected to enzyme digestion. We succeeded in propagating five of seven distinct primary breast TIL (71%) **(Table II).** Taking in consideration that enzymatic digestion provides low cell yields and may lead to significant damage of infiltrating lymphocytes, this approach may be useful for isolation of TIL from small breast samples, or needle biopsies. The T cell phenotype of the TIL was determined together with the expression of HLA-A2 **(Table I and II).** 12/20 cultures were HLA-A2 which is higher (60%) than the range of HLA-A2 expression in the human population. Overall $CD4^+$ T cells predominated in 3 TIL cultures (21%) while $CD8^+$ cells were more numerous in 9 cultures (64%). In two cultures, both $CD4^+$ and $CD8^+$ cells failed to reach the level of 50%. A differrent T cell phenotype was observed for breast TAL. Five of

six proliferated as predominantly CD4+ cells. HLA-$A2^+$ (3/6) TAL samples (50%) were HLA-$A2^+$ which is in the range observed in the population.

Cytolytic activity of breast TIL and TAL

Autologous tumors in sufficient numbers and purity to perform CTL assays were obtained from four primary breast cancers (No. 1, 2, 3, 4) and four patients with metastatic tumors (1, 2, 3 and 6). The specificity targets for this study consisted of the tumors autologous with TAL-1 ($A2^+$) and TAL-6 ($A2^-$) respectively, which were lysed at low levels by the autologous TIL, and the breast lines SKBR3 ($A2^-$) and SKBR3.$A2^+$. TIL-3, TAL-1, and TAL-3 showed preferential lysis of autologous tumors **(Figures 1A–D)**. TAL-3 showed similar levels of lysis of autologous tumor and of the SKBR3.A2 line but lower levels of lysis of HLA-$A2^-$ targets suggesting the presence of a shared Ag between autologous tumor and SKBR3.A2 cells. Although expression of TAL-3 tumor lysis did not require in vitro stimulation, for TAL-1 this required either stimulation with OKT3 mAb, or stimulation with OKT3 mAb plus autologous tumor **(Figure 1C-D)**.

TIL-1 and TIL-4 as well as TAL-2 showed non-specific lysis. TIL-2 and TAL-6 showed borderline lysis ($\leq$ 5%) of autologous tumors at an E:T ratio of 20:1. Three other TIL (No. 6, 8 and 9) for whom the autologous tumor was not available, were tested in the same experiment against a panel of three breast tumors and the NK targets K562. TIL-6 and TIL-8 showed very high lysis of K562 cells (>50%) and low lysis of all the tumor targets (<20% at E:T = 20:1) (data not shown). We tentatively concluded that these TIL do not exhibit specific autologous tumor lysis. TIL-9 showed significantly lower lysis of K562 (<40% in the same conditions) suggesting that specific tumor lysis may be possible. Overall, 3 out of 8 HLA-$A2^+$ breast TIL/TAL tested showed preferential recognition of autologous tumors **(Table I)**.

Since these results suggest that tumor specific CTL may either be present in low numbers, and/or anergic, we examined the effect of stimulation with OKT3 mAb in the presence of IL-2 on the expression of autologous tumor lysis by TAL-1. These results are shown in **Figure 2**.

Stimulation with OKT3 mAb of TAL-1, containing both $CD4^+$ and $CD8^+$ cells led to a minimal increase of the target lysis. OKT3 stimulation was most effective when separated $CD8^+$ cells were used **(Figure 2 line D).** The biggest difference was seen in the long CTL assay (20 h), indicating, as previously suggested, low-frequency tumor specific CTL (18).

The pattern of lysis of breast TIL that lacked specificity for autologous tumor is shown for two patients. TIL-4 lysed minimally the autologous tumor and marginally two breast targets (allogeneic tumor and SKBR3) but showed significantly higher lysis of both K562 and Daudi (MHC-I negative) cells suggesting that they express a NK/LAK like activity **(Figure 3).** TAL-2 lysed the autologous tumor ($A2^+$), the freshly isolated allogeneic tumor associated with TAL-1 ($A2^+$) and in some experiments marginally SKBR3 **(Figure 4A, B, D).** In contrast these breast TIL showed higher lysis of LAK targets but not of the tumor targets. They also lysed C1R.A2.HER-2 cells and C1R.A2 cells which express only HLA-A2 or HLA-A2 and HER-2 respectively **(Figure 4A, C).**

Of interest, isolated $CD8^+$ CTL from TAL-2 although exhibiting similar levels of autologous tumor lysis with unseparated TAL-2 showed significantly lower levels of lysis of C1R.A2.HER-2. These levels were similar with levels of lysis of C1R.A2 cells which do not overexpress HER-2 **(Figure 4C).** It should be mentioned that this type of lysis does not appear like the regular LAK activity. Most LAK cells lyse, in addition to Daudi, autologous or allogeneic tumors. Also they require significantly higher levels of IL-2 in culture to express this type of activity. TAL-1, -2, and 3 were associated with a $HER\text{-}2^{hi}$ tumor. Although TAL-2 lysed an allogeneic $HER\text{-}2^{hi}$ tumor, they failed to lyse SKBR3.A2 cells. The reasons for these differences are not yet known, but several possibilities are currently under consideration: (a) reduced levels of MHC-I and adhesion molecules; (b) absence of the tumor Ag recognized by the TIL as a consequence of immunoselection.

Breast TIL and TAL recognize CTL epitopes formed by HER-2 peptides

Tumor reactive CTL have been reported to recognize peptides derived from tumor Ag. Recent studies have reported that breast CTL can recognize: **(a)** the core peptide from the MUC-1 in both MHC-restricted and non-restricted fashion (11), **(b)** the MAGE-1 melanoma Ag (12); and **(c)** a HER-2 epitope defined by the peptide GP2 (654-662) (13). Since both TAL-1 and TAL-2 were associated with HER-2^{hi} tumors and lysed autologous tumor, we investigated their ability to recognize all the HER-2 peptides recently reported to reconstitute the activity of ovarian TIL (E75, C85, E90 and E89) and breast TIL (GP2/F53). The results are shown in **Figure 5**. Both TAL recognized these peptides, suggesting that **(a)** multiple epitopes from HER-2 constitute the targets of these CTL; **(b)** HER-2 peptides constitute shared Ag for breast and ovarian cancer. Among the 6 TAL and TIL lines studied four lines (TAL-1, -2, as well as TIL-1, -2) showed specific recognition of the CTL epitope defined by HER-2 peptide E75, (369-377).

Expression of HER-2 on tumor cells of patients whose TIL/TAL did not recognize immunodominant epitope E75 (TIL-3 and TAL-3) was also investigated. Autologous tumors overexpressed HER-2 (data not shown) indicating that lack of recognition of peptides could not be explained by low level expression of HER-2 protein. Comparison of the CTL activity levels indicates that these TAL show either significantly lower recognition of autologous tumor than of HER-2 peptides (TAL-1, -2), or higher specific recognition of autologous tumor, but low recognition of HER-2 peptides (TIL-3, TAL-3). This suggests that the level of expression of these peptides are low, but also that they may recognize other Ag. Ongoing studies in our laboratory using TIL-1, TIL-2, and TAL-1 as indicators have identified a second candidate tumor Ag derived from the Notch receptor complex (Anderson et. al. manuscript in preparation) (19).

DISCUSSION

The results presented in this study show for the first time a reproductible approach for expansion of breast TIL and TAL, with minimal intervention in terms of enzyme digestion, restimulation with autologous tumor/OKT3 mAb, and addition of exogenous cytokines. The non-enzymatic approach is complementary to the enzymatic digestion. In fact, TIL-12, -13 and -14 were isolated from samples subjected in parallel to enzymatic and non-enzymatic processing, of which only the latter was successful. We expanded in culture breast TIL from fourteen patients and TAL from 6 additional patients. Of the established TIL cultures from primary tumors, 9 were propagated from TIL isolated by enzymatic digestion, while five were propagated without enzymatic digestion. TIL from five additional patients isolated by enzymatic digestion failed to grow in culture. There was no difference in the doubling times, between the TIL and TAL populations. For TIL/TAL expansion, the availability of tumor for repeated restimulation is viewed as a critical factor (8). We have observed significant T cell proliferation in 20 of 27 cases attempted (74%) as well as in TIL from small tumors in which restimulation with autologous tumor cells could not be performed. Our results show that breast CTL-TIL can be propagated with high efficiency even from small tumor samples after the original stimulation with autologous tumor and OKT3 mAb and expanded into large numbers.

Fourteen TIL established in culture, grew as predominantly $CD4^+$ or $CD8^-$ cells. Only one of the fourteen breast TIL cultures (TIL-3) expanded as predominatly $CD4^-$, $CD8^+$. This line contained a low number of $CD16^+$ cells but high $TCR\gamma\delta^+$ cells. The presence of $TCR\gamma\delta^+$ cells on lymphocytes populations of breast cancer patients has been reported (20). All six TAL from distinct patients were successfully expanded in culture. This suggests that breast CTL-TIL can be successfully propagated in vitro in the presence of low doses of IL-2 and TNF-α as shown by our previous studies with ovarian TIL (15). IL-2 and IL-12 was less effective than IL-12 and TNF-α in propagating TIL (Anderson, unpublished observations). This may be useful for both adoptive cellular therapies and tumor antigen identification for cancer vaccines.

The samples were collected at random and cultured without knowing the HLA-type of the donor. Overall, 9 of the 14 growing TIL were HLA-A2 (64%). Although the sample size is small, this suggests a proliferative advantage for T cells infiltrating primary breast tumors in HLA-A2+ patients. In TAL, HLA-A2$^+$ cultures were three of six (50%). Additional studies are required to clarify whether expression of HLA-A2 associates with immunosurveillance or protection against breast cancer spread.

The phenotype of cultured TIL showed a different picture from the phenotype of TAL. 9/14 primary TIL expanded as predominatly CD8$^+$ lines (with $\geq$ 50% CD8$^+$ cells in the population) while 0/6 TAL were predominatly CD8$^+$. Since both TIL and TAL were cultured in the same conditions without regard of the source, the different outcome in the phenotypes may suggest an active priming and stimulation *in situ* of CD8$^+$ in primary tumors. Of interest, the percentage of CD8$^+$ cells was higher in cultured TIL isolated without enzymatic digestion (4/5, 80%) than in samples propagated after enzymatic digestion (5/9, 56%). Since the average of CD8$^+$ in the peripheral blood ranges between 25 - 33%, this suggests an active recruitment of CD8$^+$ cells at the primary tumor site, compared with the pleural effusion/ascitic TAL, where contamination from passenger lymphocytes may be higher.

The limited use of stimulation with tumor and OKT3 mAb in the presence of moderate concentration of IL-2 leads to breast CTL that express tumor lysis with higher frequency than some (5, 9-10) but lower than other studies (7, 8). We found autologous tumor lysis in seven of eight TIL plus TAL tested. The criteria for definition of specificity used in this study were similar with criteria used in our previous studies in ovarian cancer (21). We found preferential autologus tumor lysis only in three of eight breast TIL plus TAL tested (37%). However, such criteria may suffer from inherent limitations due to the presence of shared Ag, the differences in target lysability, the lack of information on the HLA-phenotype, and the limited amount of autologous tumor available.

Recent reports using cytotoxicity studies have established the presence among breast TIL/TAL of lymphocytes specifically recognizing

autologous tumors. Linehan et al successfully expanded breast TAL using low dose IL-2 and repeated stimulation with OKT3 mAb and tumor from six of six specimens (8). These TAL showed preferential lysis of the autologous tumor. Baxevanis et al successfully expanded breast TIL from ten patients and TAL from two patients, and after two cycles of stimulation with autologous tumor and moderate doses of IL-2; 8/12 TIL showed preferential lysis of autologous tumors (7). Specific recognition of autologous tumor was also documented by testing for cytokine secretion (5). Dadmarz et al reported that a number of $CD4^+$ TIL recognized autologous tumor in a MHC-class II restricted fashion. Specific recognition by $CD4^+$ T cells was shown by determining tumor-specific cytokine release (10).

The minimal use of autologous tumor and OKT3 stimulation only at the initiation of the culture allows to analyze tumor recognition in parallel with that of peptide epitopes. This approach may be also useful to identify the dominant peptide specificities of tumor associated CTL. The HER-2 peptides recognized may reflect Ag that have been stimulatory during tumor progression, but either not sufficiently strong to elicit a curative response, or novel tumor variants were selected. These variants may not express these Ag in sufficient amount to sensitize CTL for killing, or may express other Ag. It should be mentioned that all tumor Ag reported to date are recognized with low affinity (17, 22).

CTL recognition of ovarian and breast tumors together with that of E75 and GP2 respectively was associated with HER-2 overexpression (13, 17, 23, 24). We observed that in addition to GP2 other HER-2 peptides are also recognized. This suggests that multiple HER-2 epitopes may be presented to CTL. This raises the question as to whether these CTL epitopes are always present on the tumors. TAL-1 and TAL-2 were characterized by specific recognition of several HER-2 epitopes and lysis of autologous tumors. Conversely, TIL-3 and TAL-3 lacked specific recognition of HER-2 peptides E75, GP2 and C85, despite preferential autologous tumor killing and $HER\text{-}2^{hi}$ expression on tumor cells. This raises the questions as to whether: (a) these CTL recognize Ag that are not present on the autologous tumors at the time of collection but at an earlier

stage; (b) the antigens are present on the tumor but their density is significantly below the density required to sensitize a CTL lytic response. The T2 reconstitution assay has the advantage of amplifying the Ag density by several logs. (c) tumor recognition may be impaired by blocking molecules. The Ag may be present on the tumor but they cannot induce a CTL response since breast tumors lack co-stimulatory molecules (e.g. of the B7 family). The lack of IL-2 expression in primary breast TIL has been recently reported (4). Conversely, if other co-stimulatory molecules are involved, differences in Ag processing betwen professional APC and tumor cells related to precursor stability may lead to presentation of different stimulatory peptides from the same protein. Ongoing studies in our laboratory aim to address these points.

Identification of peptides recognized among the pool of candidate epitopes may allow development of vaccines that can be used to amplify a CTL response to breast cancer earlier, when the tumor is small. Conversely identification of specificities amplified after repeated stimulation of TIL with autologous tumor (when available) may be useful for adoptive therapies with CTL-TIL plus cytokines. These therapies may be more suitable for patients with advanced disease who are less likely to respond to cancer vaccination.

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FIGURE LEGENDS

Figure 1. Target recognition by: **(A)** TIL-3; **(B)** TAL-3; **(C)** TAL-1 stimulated with OKT3 mAb, **(D)** TAL-1 stimulated with autologous tumor followed by OKT3 mAb. (▲) autologous tumor; (Δ) HLA-A2$^-$ freshly isolated allogeneic tumor; (O) SKBR3 (HLA-A2$^-$), (❑) SKBR3.A2 (HLA-A2$^+$) TIL-3 lysed significantly K562 only at 40:1 E:T.

Figure 2. Target recognition by TAL-1, stimulated in vitro with: **(a)** Bulk culture, no stimulation; **(b)** bulk culture, stimulated with OKT3 mAb; **(c)** CD8$^+$ TAL-1, unstimulated; **(d)** CD8$^+$ TAL-1 stimulated with OKT3 mAb. **(A)** 5 h assay, **(B)** 20 h assay, ( ) Autologous tumor, ( ) K562 cells; E:T ratio was 10:1

Figure 3. Cytolytic activity of TIL-4: **(A)** after 3 weeks in culture; **(B)** isolated CD8$^+$ cells after 5 weeks in culture. Freshly isolated tumor (●) was available only for the experiment shown in pannel A.; (O) Allogeneic freshly isolated HLA-A2$^+$, HER-2^{hi} tumor; (❑) C1R.A2; (■) C1R.A2.HER-2hi cells; (Δ) SKBR3 (HLA-A2$^-$, HER-2^{hi}), (+) K562, (X) Daudi. Results were determined in a 5h CR 51 release assay.

Figure 4. **(A,C)** Recognition of autologous tumor by TAL-2; **(A)** after one week in culture, unseparated TAL; **(B)** TAL-2 stimulated with OKT3 mAb one week before the CTL assay; **(C)** CD8$^+$ cells isolated two weeks after initiation of the culture, cultured for one additional week under the same conditions for a total of three weeks. **(D)** TAL-2 re-stimulated with OKT3 mAb cultured for a total of five weeks. (O) autologous tumor () allogeneic, HLA-A2$^+$, HER-2^{hi} breast tumor, (❑) C1R.A2 cells, (■) C1R.A2 HER-2 cells (transfected with and expressing HER-2^{hi}), (Δ) SKBR3 cells; (+) K562; (X) Daudi; Freshly isolated autologous tumor was available only for the experiments shown in **(A)** and **(C)**. The lysis of SKBR3.A2 (HLA-A2$^+$, HER-2^{hi}) was 0.0% at both 40 and 60:1 E:T ratios, suggesting that these CTL do not recognize HLA-A2 associated epitopes on this tumor.

Figure 5. Recognition by breast TAL of HER-2 peptides; **(A)** Effectors were TAL-1 stimulated in culture with autologous tumor. E:T ratio was 10:1. Similar results were obtained at E:T, 40:1. One representative experiment of two performed over a 4 weeks period is shown. **(B)** Effectors were $CD8^+$ TAL-2 expanded in culture by stimulation with OKT3 mAb. Peptides were used in the assay at a concentration of 10 μg/ml. F53 is the Synthetic Antigen Laboratory designation for the peptide GP2 (13). Recognition of E75 by TAL-3 at 20:1, E:T was as follows (no peptide = 14.8, E75, 14.8).

Table II Surface Phenotype and Growth Characteristics of Freshly Isolated Breast TIL Separated Without Enzyme Digestion

TIL	HLA-A2	Phenotype CD4+	Phenotype CD8+	Days in Culture	Cells ($x10^6$)[a]
Breast					
TIL-10	+	16	89	14	10.5
TIL-11	+	8	95	14	12.6
TIL-12	-	29	59	21	34.0
TIL-13	+	4	61	7	7.4
TIL-14	+	37	46	11	4.7
Ovarian[b]					
TIL-OVA-12	-	57	37	10	25.6
TAL-OVA-13	+	76	30	14	17.9

[a]Breast tumor samples <0.5 cm were cut in small pieces and cultureD in complete RPMI 1640 containing 25 U/ml IL-2 + 100 U/ml TNF-α. OKT3 mAb was added to the culture after 24 h, and the culture was continued for the indicated number of days. The amount of lymphocytes could not be determined in the original sample.

[b]Phenotype and cell numbers obtained from two ovarian T-cell lines, one isolated from a primary/solid tumor and the second from malignant ascites are shown for comparison purposes.

Table I. Phenotypic and functional characteristics of breast TIL and TAL cultures[a].

			% positive		Tumor Lysis		
	Origin	HLA-A2	CD4	CD8	Auto	Allo	Specific
A.	*Tumor Infiltrating Lymphocytes*						
1.	Primary	+	8	95	±	+	-
2.	Primary	+	4	95	+	+	-
3.	Primary	+	4	30	+	+	+
4.	Primary	+	75	25	-	+	-
5.	Primary	+	30	64	n	n	n
6.	Primary	-	69	30	n[c]	+	-
7.	Primary	-	81	27	n	n	n
8.	Primary	-	12	85	n[c]	+	-
9.	Primary	-	33	67	n[c]	+	-
B.	*Tumor Associated Lymphocytes*						
1.	Pleural effusion	+	65	35	+	+	+
2.	Pleural effusion	+	60	36	+	+	-
3.	Pleural effusion	+	66	33	+	+	+
4.	Pleural effusion	-	74	33	n	n	n
5.	Ascites	-	45	29	n	n	n
6.	Ascites	-	n	48	±	+	-

[a]The doubling time tumors determined for weeks 2 and 3 was three days excepting TIL-9, TAL-4 (4 days), TIL-4, -8 (5 days), TIL-5 (>7 days). [b](+) indicates that the corresponding TIL/TAL lysed: (+) better the autologous tumor than two other breast tumors of which one was a freshly isolated breast tumor; (-) better allogeneic breast tumors, K562 and Daudi cells than the autologous tumor. (n) not performed, (±) borderline (≤5%) lysis. 5 h assay but higher (10-15%) in 20 h assay. [c]Autologous tumor not available.

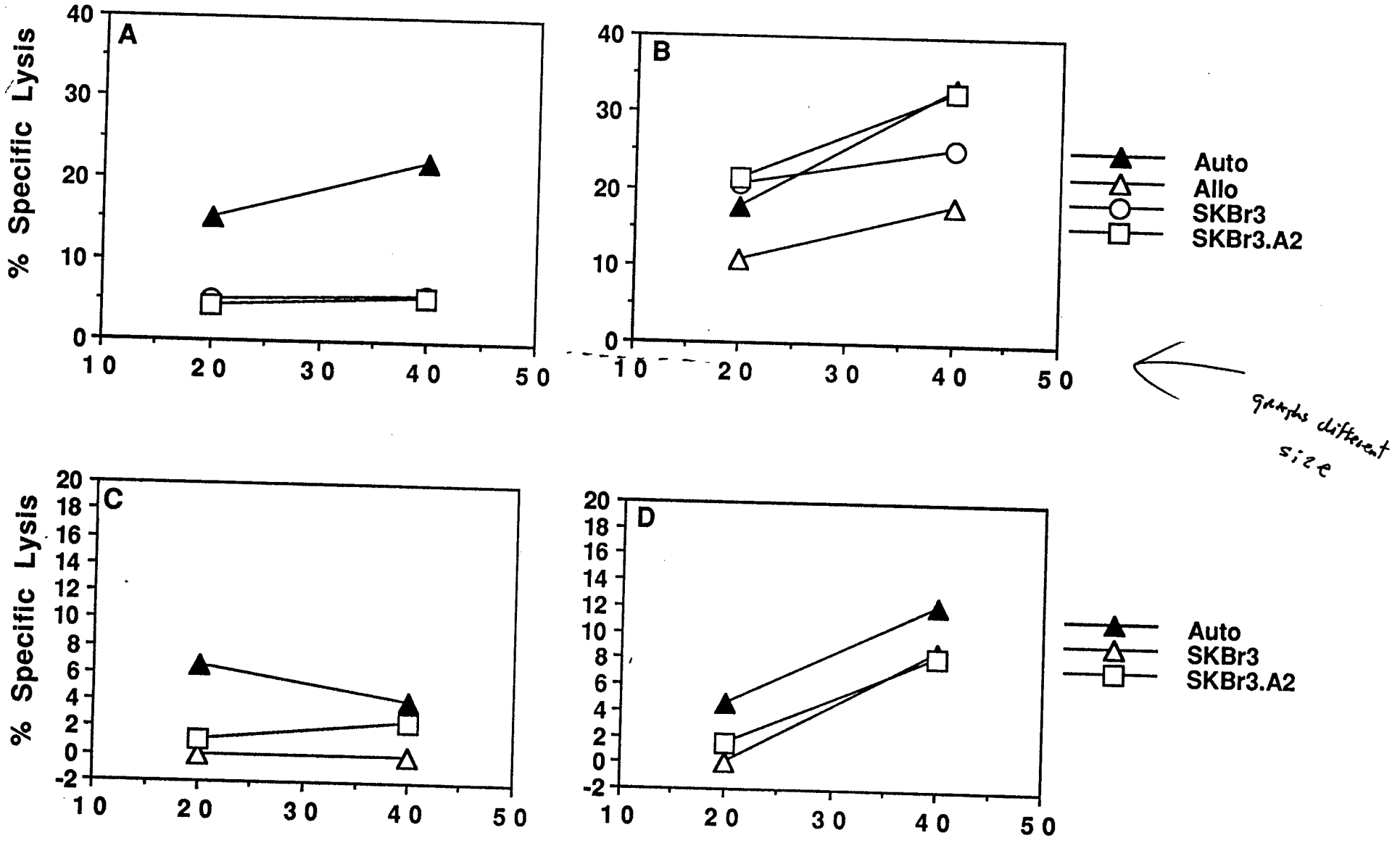
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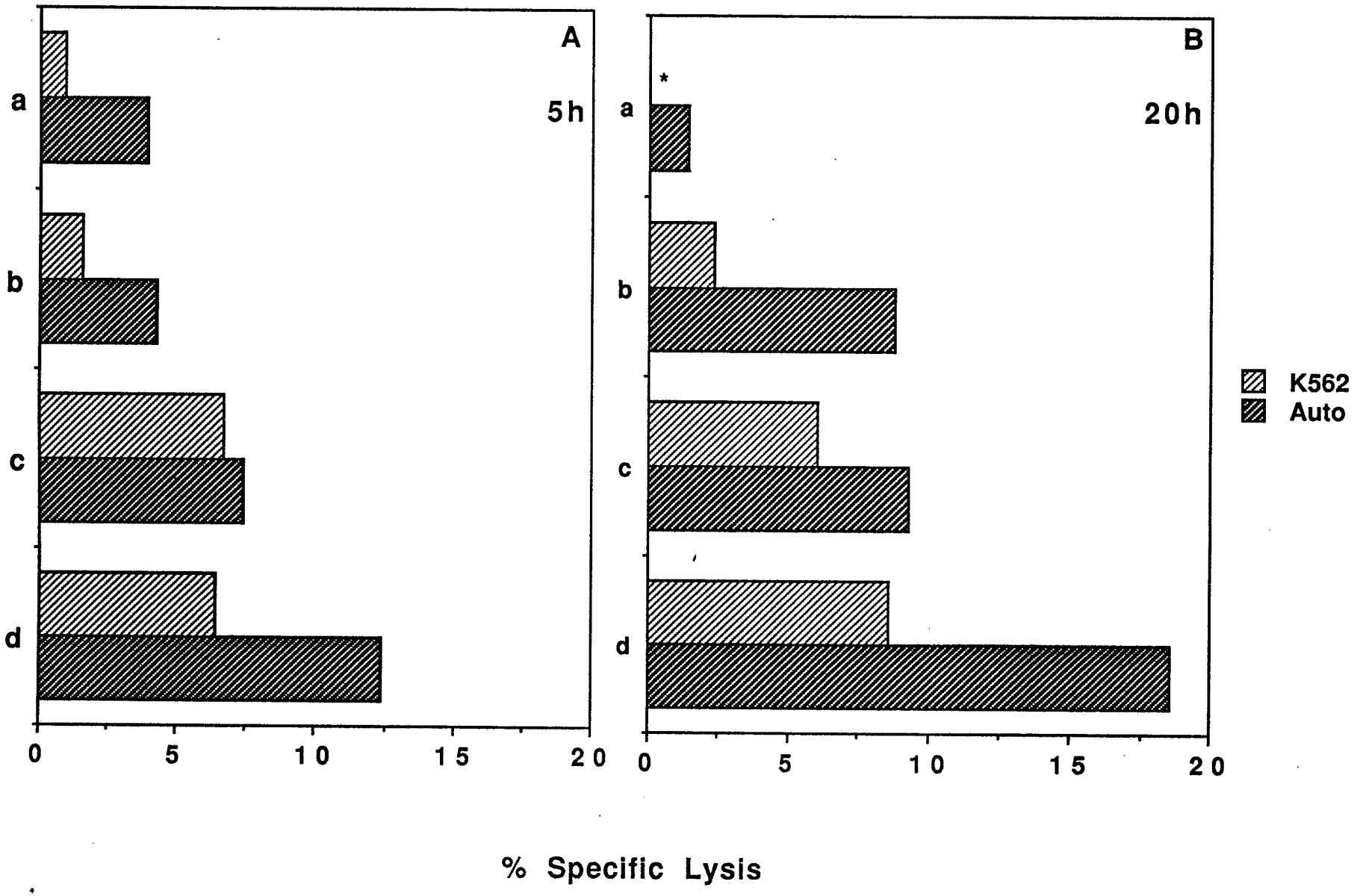
Figure 1

Figure 2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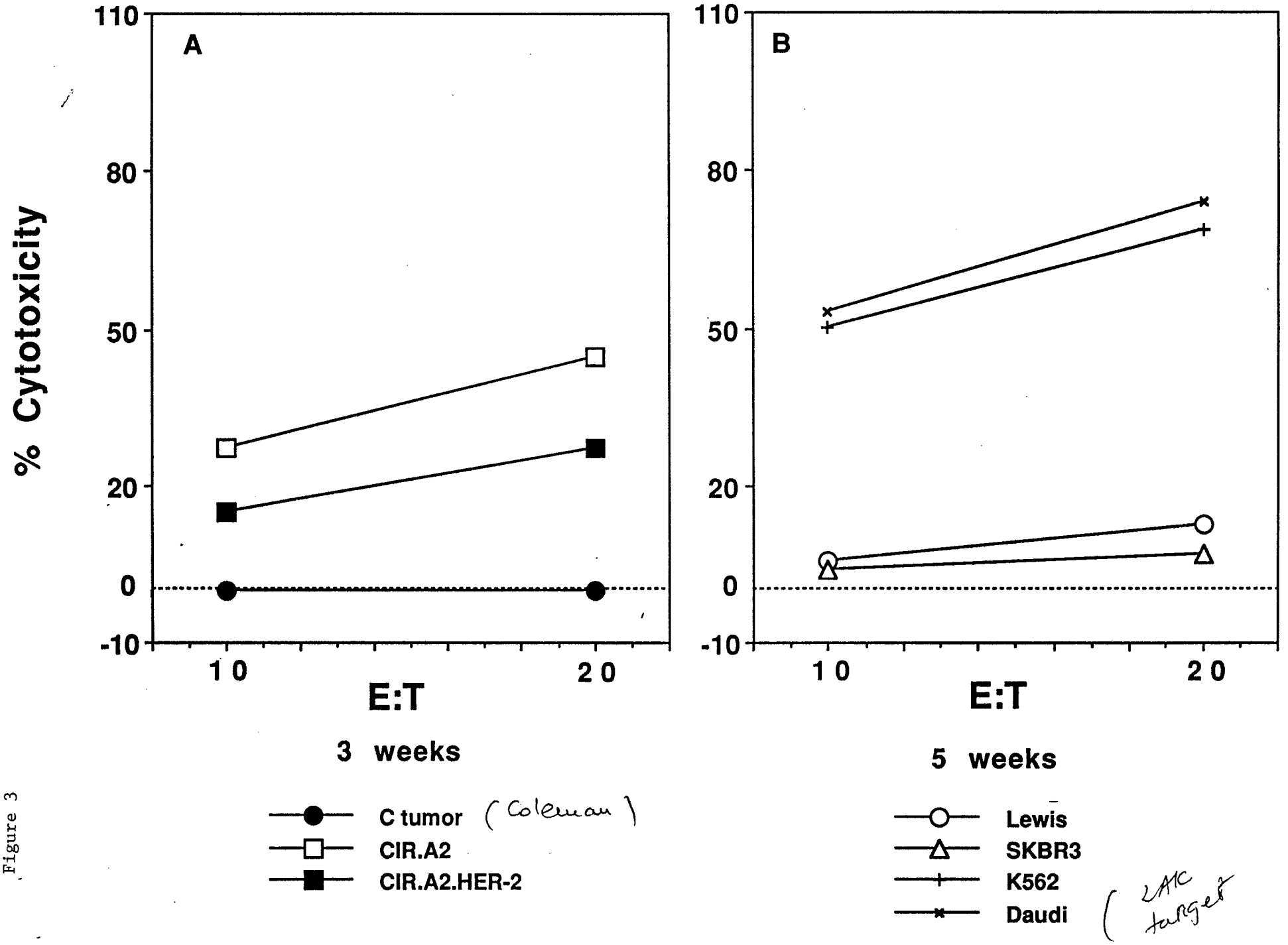
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Figure 3

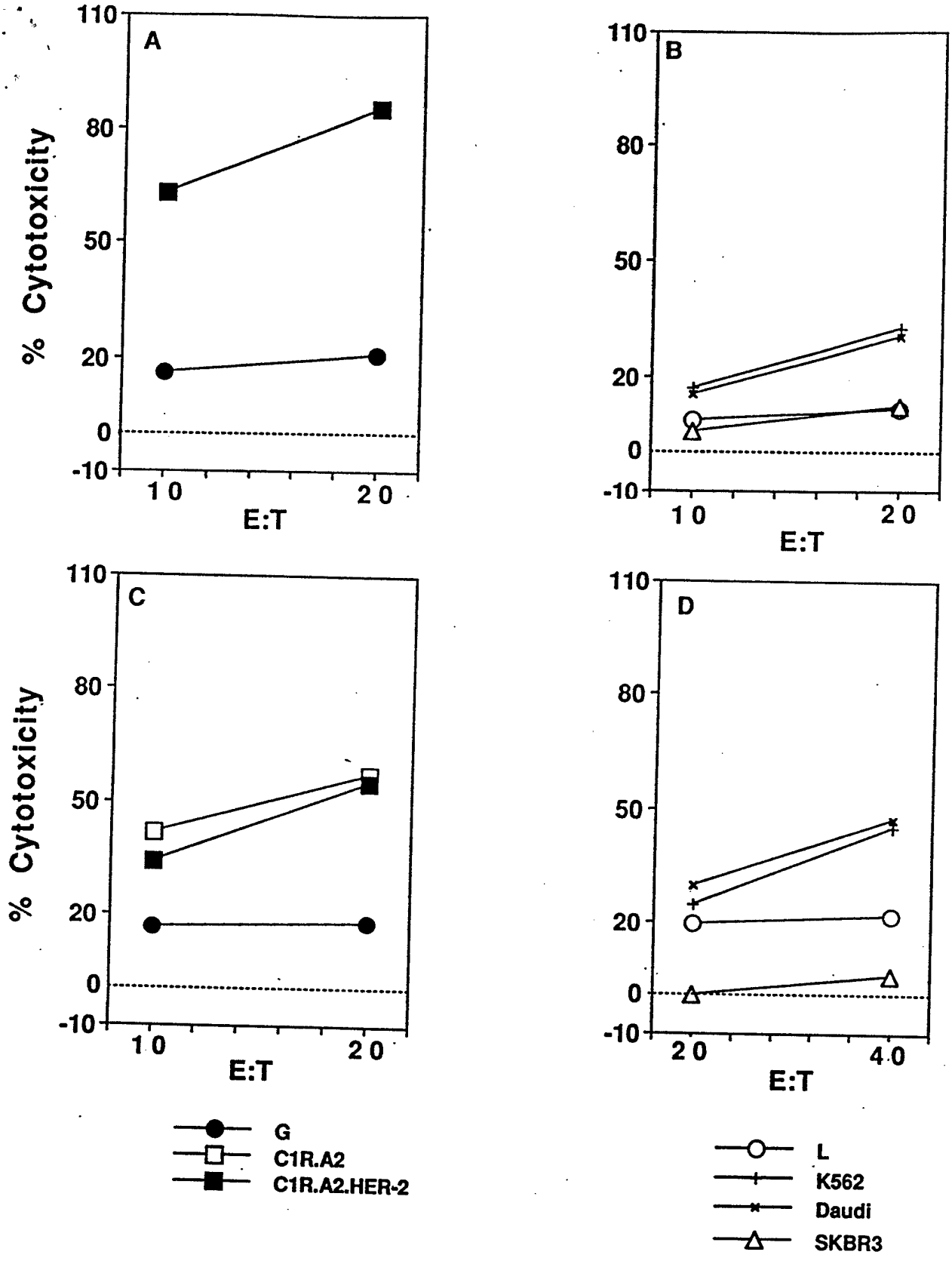
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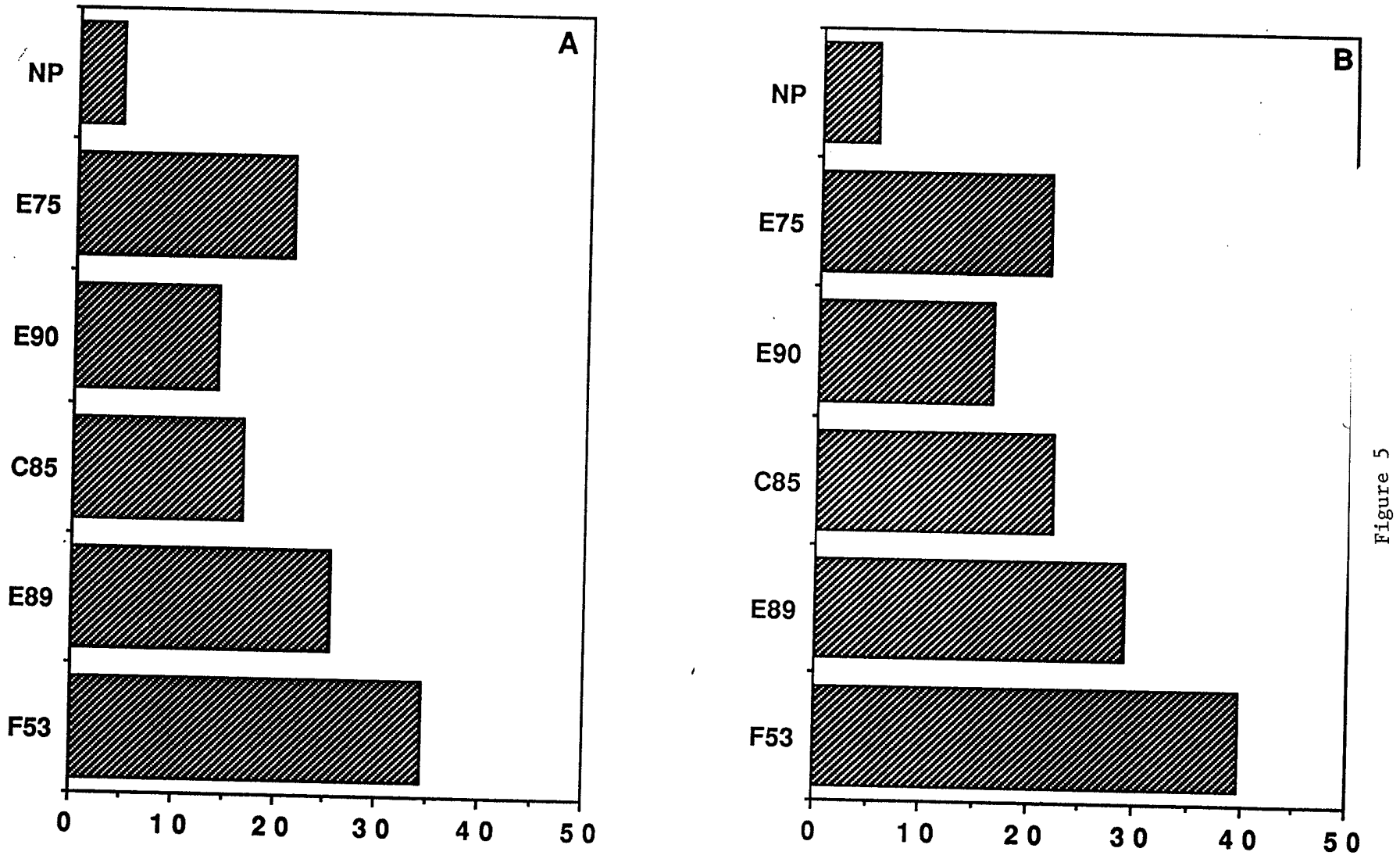
Figure 4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% Specific Lysis

Figure 5

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[a]Breast tumor samples <0.5 cm were cut in small pieces and cultures in complete RPMI 1640 containing 25 U/ml IL-2 + 100 U/ml TNF-α. OKT3 mAb was added to the culture after 24 h, and the culture was continued for the indicated number of days. The amount of lymphocytes could not be determined in the original sample.

[b]Phenotype and cell numbers obtained from two ovarian T-cell lines, one isolated from a primary/solid tumor and the second from malignant ascites are shown for comparison purposes.

Investigational New Drugs
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Potent Induction of Human Cancer Cell Uptake of Chemotherapeutic Drugs by *N*-myristoylated Protein Kinase C-α (PKC-α) Pseudosubstrate Peptides through a P-Glycoprotein-Independent Mechanism

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Key words: protein kinase C (PKC), intrinsic drug resistance, human colon cancer, multidrug resistance (MDR), MDR reversal, *N*-myristoylated pseudosubstrate peptides.

Summary

Phorbol-ester protein kinase C (PKC) activators and PKC isozyme overexpression have been shown to significantly reduce intracellular accumulation of chemotherapeutic drugs, in association with induction of multidrug resistance (MDR) in drug-sensitive cancer cells and enhancement of drug resistance in MDR cancer cells. These observations constitute solid evidence that PKC plays a significant role in the MDR phenotype of cancer cells. PKC-catalyzed phosphorylation of the drug-efflux pump P-glycoprotein was recently ruled out as a contributing factor in MDR. At present, the sole drug transport-related event that has been identified as a component of the role of PKC in MDR is PKC-induced expression of the P-glycoprotein-encoding gene *mdr1*. The objective of this study was to test the hypothesis that PKC can modulate the uptake of chemotherapeutic drugs in cancer cells independently of P-glycoprotein. We analyzed the effects of selective PKC activators/inhibitors on the uptake of radiolabeled cytotoxic drugs by cultured human colon cancer cells that lacked P-glycoprotein activity and did not express the drug efflux pump at the level of message (*mdr1*) or protein. We found that the selective PKC activator 12-O-tetradecanoylphorbol-13-acetate (TPA) significantly reduced uptake of [^{14}C] Adriamycin and [^{3}H] vincristine in human colon cancer cells devoid of P-glycoprotein activity, and that PKC-inhibitory *N*-myristoylated PKC-α pseudosubstrate synthetic peptides potently and selectively induced uptake of the cytotoxic drugs in the phorbol ester-treated and nontreated colon cancer cells. TPA treatment of the cells did not induce expression of either P-glycoprotein or its message *mdr1*. In contrast with [^{14}C] Adriamycin and [^{3}H] vincristine uptake, [^{3}H] 5-fluorouracil uptake by the cells was unaffected by TPA and reduced by the PKC-inhibitory peptides. These results indicate that PKC activation can significantly reduce the uptake

of multiple cytotoxic drugs by cancer cells independently of P-glycoprotein, and that *N*-myristoylated PKC-α pseudosubstrate peptides potently and selectively induce uptake of multiple cytotoxic drugs in cultured human colon cancer cells by a novel mechanism that does not involve P-glycoprotein and may involve PKC isozyme inhibition. Thus, *N*-myristoylated PKC-α pseudosubstrate peptides may offer a basis for the development of agents that reverse intrinsic drug resistance in human colon cancer.

Introduction

In recent years, the seminal observation in 1988 that phorbol esters induce multidrug resistance (MDR) in human breast cancer cells [1] has spawned investigations into the potential role of the phorbol-ester tumor promoter receptor family protein kinase C (PKC) [2] in MDR (reviewed in 3). In support of an integral role for PKC in MDR, phorbol esters have been shown to induce a defect in intracellular drug accumulation accompanied by MDR in diverse cancer cell lines [1,3-6], and increased expression of the isozyme PKC-α has been noted in a number of drug-selected MDR cancer cell lines [3,7-9]. The report that PKC phosphorylates P-glycoprotein [4] and the identification of the phosphorylation sites in the linker region of the drug transporter [10] led to the hypothesis that the contribution of PKC to MDR revealed by phorbol ester effects was due to PKC-catalyzed P-glycoprotein phosphorylation. This hypothesis was recently disproved by two definitive reports that characterized human P-glycoprotein mutants containing specific alterations at the phosphorylation sites. The mutations were shown to have only minor or negligible effects on the expression and transport activity of P-glycoprotein [11,12]. The authors speculated that the contribution of PKC to MDR that is associated with altered drug transport may be restricted to induction of the *mdr1* gene, which encodes P-glycoprotein [12].

We previously reported that *N*-myristoylated PKC-α pseudosubstrate peptides partially reverse the P-glycoprotein-dependent MDR phenotype of human breast cancer MCF7-MDR cells in association with PKC-α inhibition and restoration of intracellular drug accumulation [13]. We also showed that the MCF7-MDR cells are not cross-resistant to the PKC-inhibitory peptides [13], and that the peptides are not P-glycoprotein substrates [14]. In this report, we show that phorbol-ester tumor promoters induce a significant reduction in chemotherapeutic drug

uptake which is opposed by a potent induction of drug uptake by *N*-myristoylated PKC-α pseudosubstrate peptides in human colon cancer cells that do not detectably express P-glycoprotein at the level of either protein or message (*mdr*1) and do not exhibit altered drug uptake in the presence of P-glycoprotein modulators. Our results indicate that the contribution of PKC to MDR extends to effects on P-glycoprotein-independent drug transport mechanisms.

Materials and Methods

Materials

The metastatic human colon cancer line KM12L4a [15] was maintained as a monolayer culture as previously described [5]. This cell line has no exposure to cytotoxic drugs in its history [5,15]. The human breast cancer lines MCF7-WT and MCF7-MDR were maintained as previously described [13]. Tissue culture reagents were purchased from GIBCO BRL (Gaithersburg, MD). The P-glycoprotein monoclonal antibody C219 was purchased from Signet Laboratories (Dedham, MA). Molecular weight markers and other SDS PAGE reagents were from BioRad Labs (Hercules, CA). The *N*-myristoylated synthetic peptides were synthesized and HPLC-purified to >98% purity at The M. D. Anderson Cancer Center Synthetic Antigen Facility, as previously described [13]. [^{14}C] Adriamycin (55 mCi/mmol), [^{3}H] vincristine sulfate (2 Ci/mmol), an enhanced chemiluminescence (ECL) detection kit, nitrocellulose sheets, and horseradish peroxidase-linked sheep anti-mouse and anti-rabbit Ig were purchased from Amersham Corp. (Arlington Heights, IL). [^{3}H] 5-fluorouracil (14 Ci/mmol) was obtained from DuPont NEN. Cyclosporin A was from Sandoz (East Hanover, NJ), and verapamil, phorbol esters, and all other reagents were from Sigma Chem. Corp.

Drug Accumulation Assays

The accumulation of radiolabeled drugs in human colon cancer KM12L4a cells was measured by an established method [5,13]. Briefly, KM12L4a cells were cultured on 24-well Costar plates (201 mm^2/well) at a density of 5 X 10^5 cells/well. Following a 20-24 h attachment period at 37°C, the cells were preincubated for 30 min at 37°C with test reagents under investigation for effects on drug accumulation (phorbol esters, *N*-myristoylated peptides,

verapamil, cyclosporin A). Next, the pretreated cells were incubated for 2 h at 37°C with the radiolabeled cytotoxic drug (0.1 μM [^{14}C] Adriamycin, 10 nM [^{3}H] vincristine, or 10 nM [^{3}H] 5-fluorouracil) in the continued presence of the test reagent. The drug accumulation assay was terminated by rapidly washing the cells three times with ice-cold PBS, and cells were detached from the plate by a 30-min exposure to trypsin-EDTA at 37°C. Cells were harvested, placed in vials containing 15 ml. scintillation fluid, and counted [5,13].

Western Blot Analysis

The level of P-glycoprotein expression in the cancer cell lines KM12L4a, MCF7-WT, and MCF7-MDR was measured by Western blotting [13]. Briefly, cells were collected in 2 ml of lysis buffer (20 mM Tris-HCl, pH 7.5, 15 mM ß-mercaptoethanol, 5 mM EDTA, 5 mM EGTA, 0.25 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 200 μg/ml aprotinin and 2 mM vanadate), and sonicated for 10 sec at 4°C. An aliquot was set aside at 4°C as a crude fraction, and the remainder was ultracentrifuged at 4°C for 60 mins at 35,000 rpm. The supernatant was discarded, and the pellet was resuspended in 1 ml of lysis buffer (membrane (mb) fraction). Protein concentration was determined with the BioRad protein assay kit (Hercules, CA). Samples were electrophoresed in a 7.5% SDS-PAGE gel, transferred to nitrocellulose, blocked, and probed with C219 monoclonal Ab in conjunction with an HRP-coupled secondary Ab as previously described [13]. Bands were detected using an enhanced chemiluminescence (ECL) system (Amersham). To assess loading efficiency, the nitrocellulose membranes were stripped in ß-mercaptoethanol buffer (62.5 mM Tris-HCl, pH 6.7, 100 mM ß-mercaptoethanol, 2% sodium dodecylsulfate) for 30 mins at room temperature, washed for 1 hr and then reblocked and probed with anti-ß-actin Ab (Sigma) by the procedures described above. For identification of immunospecific bands, control

Western blots that omitted the primary Ab (C219, anti-ß-actin) were run in parallel.

Northern Blot Analysis

The level of P-glycoprotein mRNA (*mdr*1) expression in the cancer cell lines KM12L4a, MCF7-WT and MCF7-MDR was measured by Northern blot analysis. Cell lines were grown to approximately 75% confluence, and mRNA was extracted as previously described [16]. mRNA was electrophoresed on a 1% denaturing formaldehyde/agarose gel, electrotransferred to GeneScreen nylon membranes (DuPont Inc., Boston, MA), and UV cross-linked using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). Membranes were pre-hybridized for 1 hr with Rapid-Hyb buffer (Amersham) and then hybridized as previously described [16]. The cDNA probes used in this analysis were a 1.38-kb *Eco*RI-cut fragment from clone pHDR5A corresponding to human *mdr*1 cDNA [17] and a 1.3-kb *Pst*I gene fragment corresponding to rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [18]. The probes were purified by agarose gel electrophoresis, recovered using GeneClean (Bio 101, La Jolla, CA), and radiolabeled by random primer-end labeling with [α-^{32}P]-deoxyribonucleotide triphosphate [19]. Hybridized blots were washed twice, dried, and exposed to Hyperfilm-MP autoradiography film (Amersham) for 2-24 hr at -70°C, and the film was developed in an automatic processor.

Statistical Analysis

The data were subjected to statistical analysis with a two-tailed Student's *t* test using Microsoft software.

Results

We previously reported that phorbol esters significantly reduce cytotoxic drug accumulation (Adriamycin, vincristine, vinblastine) in human colon cancer KM12L4a cells to 50-80% of control levels in association with induction of drug resistance (2- to 3-fold increases in the IC_{50}'s of the cytotoxic drugs) [5] and activation of PKC-α [20]. As a test of whether the phorbol ester-induced reduction of drug uptake in KM12L4a cells could involve P-glycoprotein, we examined the effects of the P-glycoprotein modulators verapamil and cyclosporin A [1,21] in this system. These P-glycoprotein-binding drugs potently inhibit the drug efflux activity of the transporter [21]. At concentrations that elicit potent P-glycoprotein inhibition, verapamil (5-25 μM) and cyclosporin A (1-4 μg/ml) failed to enhance [^{14}C] Adriamycin uptake in KM12L4a cells in the absence of phorbol esters (Fig. 1). The phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) (50 nM) significantly reduced [^{14}C] Adriamycin uptake in the KM12L4a cells as previously reported (Fig. 1) [5]. Verapamil and cyclosporin A also failed to enhance [^{14}C] Adriamycin uptake in the phorbol ester-treated cells (Fig. 1). Consistent with these results, the immunoblot analysis shown in Fig. 2 (Lanes 4,5) revealed that KM12L4a cells did not express P-glycoprotein at a detectable level whether or not the cells were treated with TPA under the conditions employed in Fig. 1. In addition, the Northern blot analysis shown in Fig. 3 revealed that the TPA-treated and untreated KM12L4a cells (Lanes 1, 2) do not express P-glycoprotein mRNA (*mdr*1). To ensure that the lack of detection of *mdr*1 in KM12L4a cells in Fig. 3 was not due to the 2 h exposure period, we also exposed the blot overnight and observed grossly overexposed bands in the MCF7-MDR lanes, a weak band in the MCF7-WT lane, and no band in the KM12L4a lanes (data not shown). These results indicate that the phorbol ester-induced reduction in

[^{14}C] Adriamycin uptake by KM12L4 cells occurs by a mechanism that is independent of P-glycoprotein-mediated drug transport.

In the Adriamycin-selected human breast cancer line MCF7-MDR, which expresses a P-glycoprotein-dependent MDR phenotype, the *N*-myristoylated PKC-α pseudosubstrate synthetic peptides NmFARKGALRQ (P1) and NmRFARKGALRQKNV (P3) (25-100 μM) [22] each induce a sharp increase in the uptake of P-glycoprotein substrates in association with inhibition of cellular PKC-α activity [13]. To test whether the effects of P1 and P3 on cytotoxic drug uptake by cancer cells extend to P-glycoprotein-independent drug transport mechanisms, we examined the effects of the pseudosubstrate peptides on drug uptake by KM12L4a cells. Fig. 4A shows that P3 induced [^{14}C] Adriamycin uptake by more than two-fold over the control value in both phorbol ester-treated and nontreated KM12L4a cells. Significant induction of [^{14}C] Adriamycin uptake was achieved at 50 and 100 μM P3 (Fig. 4A). At 100 μM, P1, which is a truncated P3 analog, also achieved significant induction of [^{14}C] Adriamycin uptake in phorbol ester-treated KM12L4a cells, resulting in a level of [^{14}C] Adriamycin intracellular accumulation that was equivalent to the level observed in control cells that were not exposed to phorbol esters (Fig. 4B). In contrast with P3, P1 (10-100 μM) did not enhance [^{14}C] Adriamycin uptake in KM12L4a cells that were not treated with phorbol esters (Fig. 4B). Thus, P3 was clearly the more potent peptide in the induction of [^{14}C] Adriamycin uptake by KM12L4a cells.

We previously reported that TPA reduces the intracellular uptake of [^{3}H] vincristine in KM12L4a cells [5]. Next, we investigated the effects of the *N*-myristoylated PKC-α pseudosubstrate synthetic peptides on [^{3}H] vincristine uptake by KM12L4a cells. Figure 5A shows that P3 significantly induced [^{3}H] vincristine uptake by more than four-fold over the control level in both phorbol-ester

treated and nontreated KM12L4a cells. An analogous although somewhat less striking significant induction of [^{3}H] vincristine uptake was achieved in KM12L4a cells by the truncated analog P1 (Fig. 5B).

In a previous report, we showed that TPA is without effect on [^{3}H] 5-fluorouracil uptake by KM12L4a cells [5]. Figure 6 confirms this and shows that neither P1 nor P3 (10-100 μM) induces [^{3}H] 5-fluorouracil uptake in phorbol ester-treated or non-treated KM12L4a cells; in fact, the peptides appear to cause a decline in KM12L4a cell uptake of [^{3}H] 5-fluorouracil.

Discussion

Although PKC-catalyzed P-glycoprotein phosphorylation has been ruled out as a contributing factor in MDR [11,12], the evidence that PKC activation contributes to MDR through effects on cellular drug uptake remains compelling. Most notable is the significant enhancement of MDR in association with a reduction in cellular drug uptake that is achieved in cancer cells by phorbol ester treatment as well as by PKC-α overexpression [1,3-6,23,24]. Plausible mechanisms underlying the involvement of PKC in MDR by modulation of cellular drug uptake fall into three categories. 1) As previously noted [12], the role of PKC in MDR may involve *mdr1* induction. This has, in fact, been demonstrated [25], but the extent to which this mechanism accounts for the enhancement of MDR that has been observed in association with increased PKC activity is not yet clear. 2) PKC isozymes may modulate P-glycoprotein function indirectly, by phosphorylating proteins that influence P-glycoprotein function. This mechanism would be analogous to the role of PKC in the regulation of the EGF receptor. Phorbol esters have profound effects on the function of the EGF receptor, which is a PKC substrate [26], yet specific mutations of the EGF receptor at sites phosphorylated by PKC and MAP kinase in response to phorbol esters appear to be without effect on EGF receptor function [27]. Recent evidence suggests that PKC may affect EGF receptor function indirectly by a mechanism involving activation of a tyrosine phosphatase [28,29]. 3) PKC activation may enhance MDR by regulating drug transport-related mechanisms that are independent of P-glycoprotein. In this report, we provide evidence that this is the case in the intrinsic drug resistance of human colon cancer KM12L4a cells.

Our results show that the phorbol-ester PKC activator TPA can significantly reduce drug uptake in a human colon cancer line devoid of P-glycoprotein activity

and expression. Because TPA is highly selective for PKC [2], these results provide strong evidence that PKC effects on drug uptake by cancer cells include mechanisms that do not involve P-glycoprotein. Furthermore, we show that PKC-inhibitory peptides potently and selectively induce cytotoxic drug uptake in the human colon cancer line by a novel mechanism that does not involve P-glycoprotein and may involve PKC isozyme inhibition.

The intrinsic drug resistance of human colon cancer generally renders chemotherapeutic intervention ineffective in the treatment of this disease, and therapeutic interventions with biological response modifiers have not substantially improved survival [30,31]. New strategies for the treatment of metastatic colon cancer are urgently needed [30,31]. Our results suggest that the *N*-myristoylated PKC-α pseudosubstrate peptides described in this report may be of value in the development of novel approaches to the reversal of intrinsic drug resistance in human colon cancer.

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Figure Legends

Figure 1. [^{14}C] Adriamycin accumulation in human colon cancer KM12L4a cells treated with cyclosporin A (CSA) (*A*) and verapamil (VP) (*B*) either alone (left half of each panel) or in the presence of 50 nM TPA (right half of each panel). In *A*, CSA concentrations were 0, 4, 2, and 1 μg/ml. In *B*, VP concentrations were 0, 25, 10, and 5 μM. 100% [^{14}C] Adriamycin accumulation is defined as the drug uptake measured in untreated control cells. Treatment conditions and the drug uptake assay are described in Methods. Under each treatment condition examined, >95% cell viability was observed at the end of the drug accumulation period by trypan blue exclusion. Each bar is the mean value of 3 experiments done in triplicate. * = $p < 0.01$ *vs.* control (untreated cells).

Figure 2. Immunoblot analysis of P-glycoprotein in lysates of TPA-treated and untreated KM12L4a cells is shown. The human breast cancer cell lines MCF7-MDR and MCF7-WT served as controls that respectively express and do not express P-glycoprotein. KM12L4a + TPA denotes a 30 min treatment of the cells with 50 nM TPA in media. Lane 1, 50 μg MCF7-MDR crude lysate protein; Lane 2, 25 μg MCF7-MDR membrane (mb) fraction; Lane 3, 25 μg MCF7-WT mb fraction; Lane 4, 50 μg untreated KM12L4a mb fraction; Lane 5, 50 μg TPA-treated KM12L4a mb fraction. The upper panel shows the result of immunoblot analysis with the P-glycoprotein-directed monoclonal Ab C219 (500 ng/ml); the bands in lanes 1 and 2 at 170 kDa are immunospecific and correspond to P-glycoprotein. The bands at 66 kDa and 130 kDa are nonspecific and also appeared in control blots where C219 Ab was omitted from the analysis. The lower panel shows the result of immunoblot analysis of the same blot with ß-actin Ab as a loading control. The methodology employed in the immunoblot analysis is fully described in Materials and Methods. The immunoblot analysis shown is representative of the results obtained in three experiments.

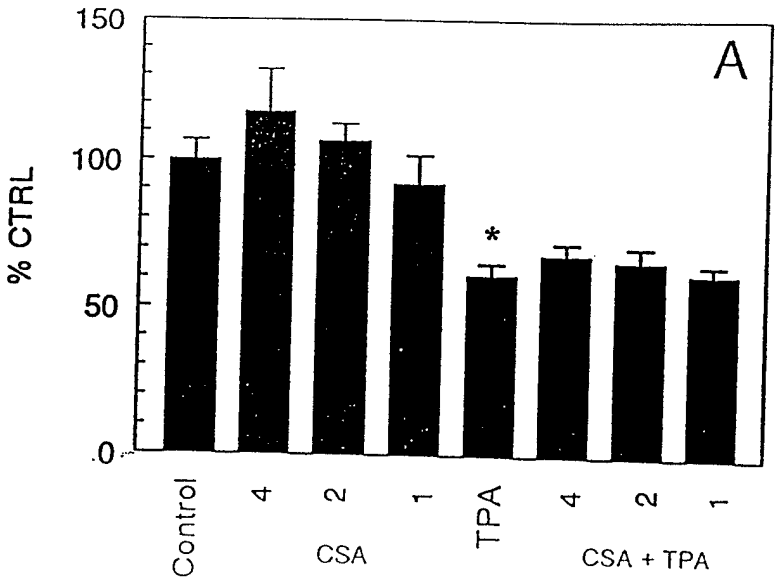
Figure 3. Northern blot analysis of *mdr*1 expression in TPA-treated and untreated KM12L4a human colon cancer cells is shown. The cell lines MCF7-MDR and MCF7-WT served as controls as described in the legend to Fig. 2. mRNA was fractionated on a 1% formaldehyde/agarose gel and probed for *mdr*1. The upper panel shows the result of Northern blot analysis probing for *mdr*1 at 2 hours of exposure. The lower panel shows the result of Northern blot analysis probing for GAPDH to assess mRNA loading in the analysis shown in the upper panel. TPA treatment of KM12L4a cells was done as described in the legend to Figure 2. Lane 1, 5 μg untreated KM12L4a mRNA; Lane 2, 5 μg TPA-treated KM12L4a mRNA; Lane 3, 5 μg MCF7-WT mRNA; Lane 4, 1 μg MCF7-MDR mRNA; and Lane 5, 5 μg MCF7-MDR mRNA. Representative results of two experiments are shown. For other details, see the legend to Figure 2 and Methods.

Figure 4. [^{14}C] Adriamycin accumulation in human colon cancer KM12L4a cells treated with the N-myristoylated PKC-α pseudosubstrate synthetic peptides P3 (*A*) and P1 (*B*) either alone (left half of each panel) or in the presence of 50 nM TPA (right half of each panel). Peptide concentrations were 0, 100, 50, and 10 μM. In *A*, each bar is the mean value of 4 experiments done in triplicate. In *B*, each bar is the mean value of 3 experiments done in triplicate. * = $p<0.01$ vs. control (untreated cells). ** = $p<0.01$ vs. cells treated with TPA alone. For other details, see the legend to Figure 1 and Methods.

Figure 5. [^{3}] vincristine accumulation in human colon cancer KM12L4a cells treated with the *N*-myristoylated PKC-α pseudosubstrate synthetic peptides P3 (*A*) and P1 (*B*) either alone (left half of each panel) or in the presence of 50 nM TPA (right half of each panel). Peptide concentrations were 0, 100, 50, and 10 μM. 100% [^{3}H] vincristine uptake is defined as the drug uptake measured in untreated (control) cells. Each bar is the mean value of 3 experiments done in triplicate.

* = $p<0.01$ vs. control (untreated) cells. ** = $p<0.01$ vs. cells treated with TPA alone. For other details, see the legend to Figure 1 and Methods.

Figure 6. [^{3}H] 5-fluorouracil accumulation in human colon cancer KM12L4a cells treated with P3 (*A*) and P1 (*B*) either alone (bars to the left of TPA) or in the presence of 50 nM TPA (bars to the right of TPA). Peptide concentrations were 100, 50, and 10 μM. "Control" denotes untreated cells, "TPA" denotes cells treated with 50 nM TPA alone, and "VP" denotes cells treated with 10 μM verapamil. 100% [^{3}H] 5-fluorouracil uptake is defined as the drug uptake measured in untreated (control) cells. Each bar is the mean value of three experiments done in triplicate. For other details, see the legend to Figure 1 and Methods.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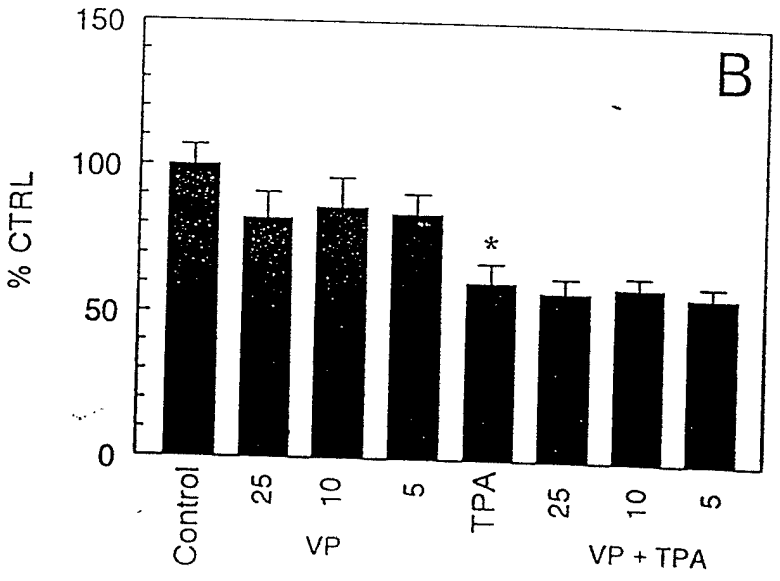
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FIGURE 1


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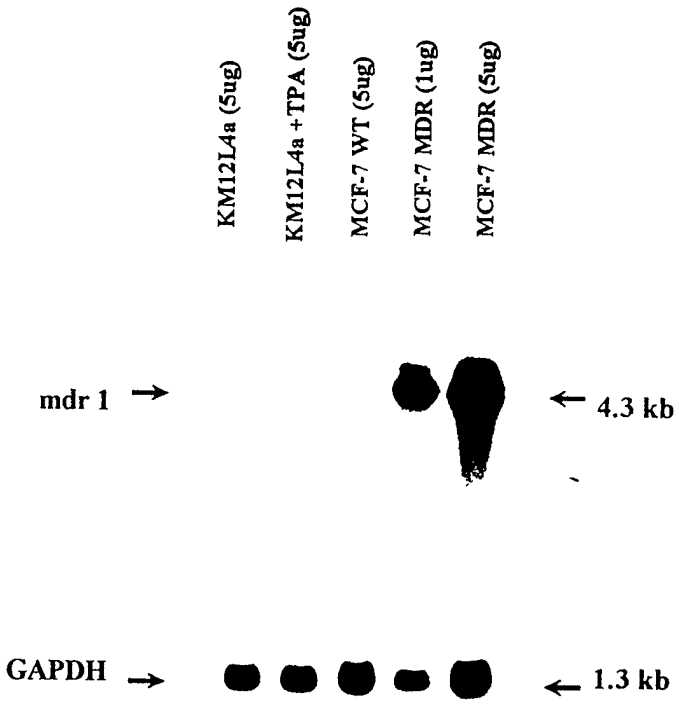
Actin (42 kD)

Figure 2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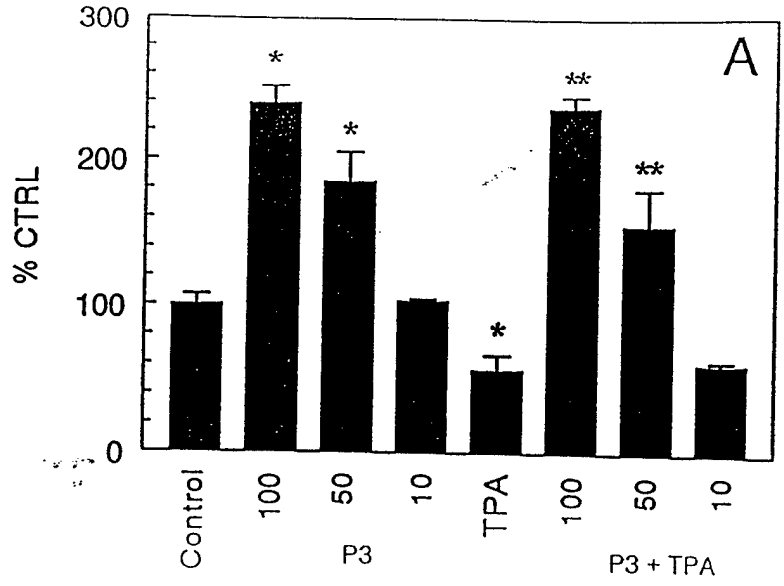
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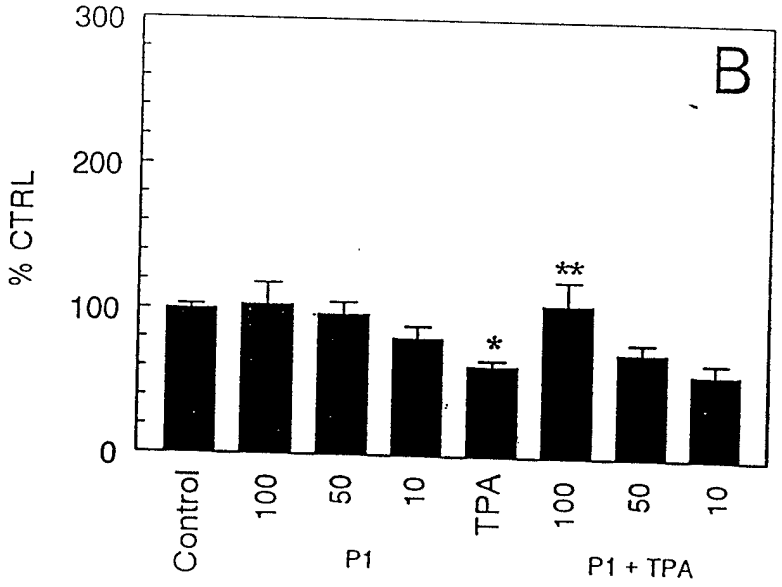
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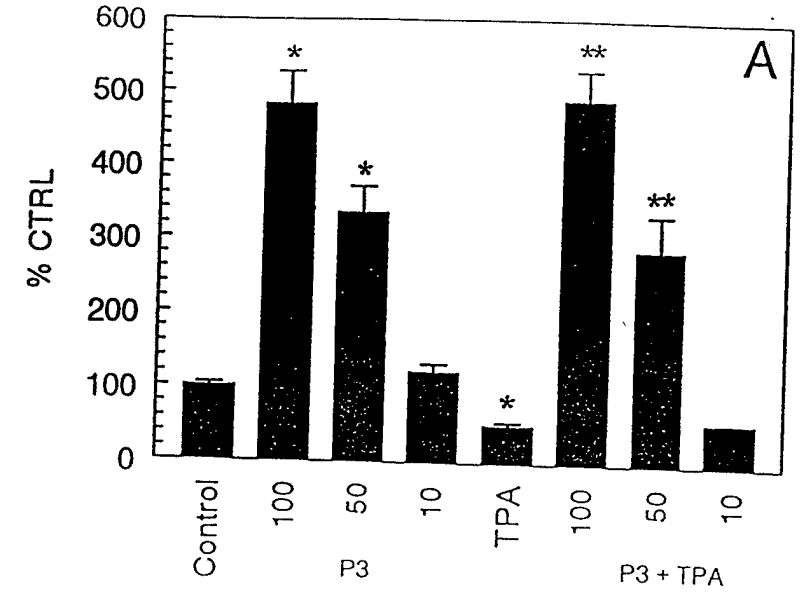
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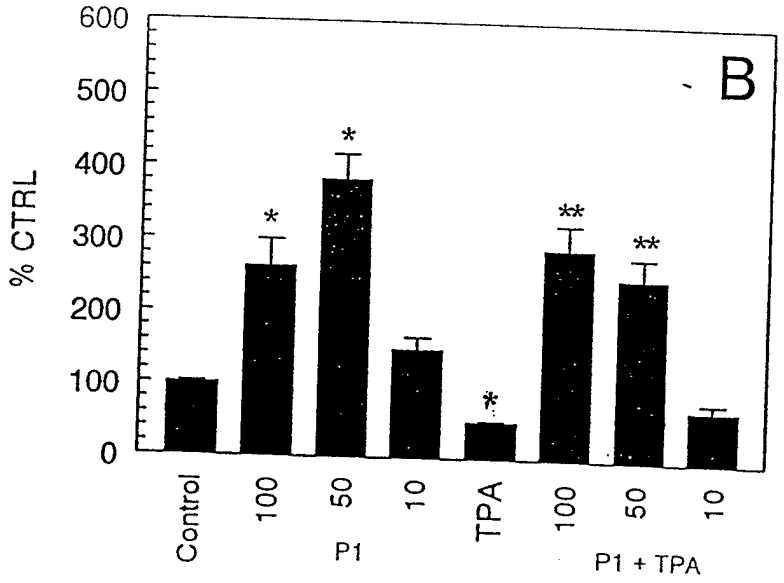
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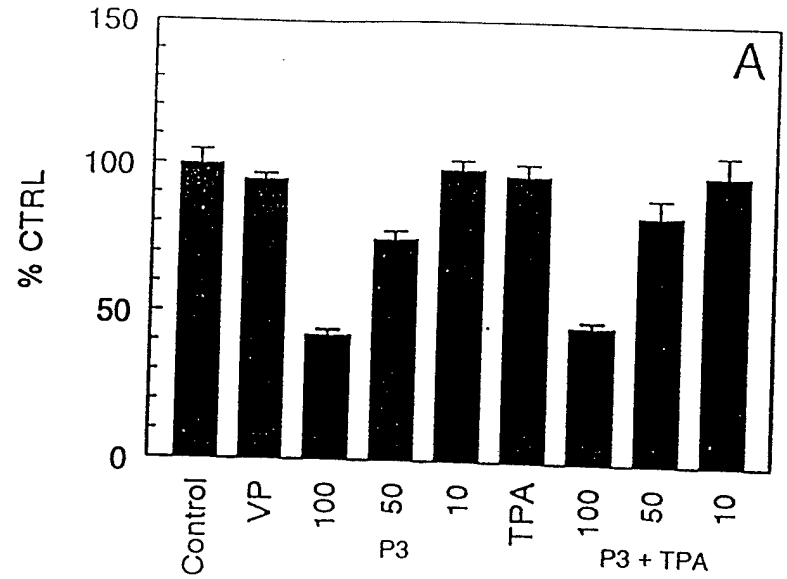
FIGURE 5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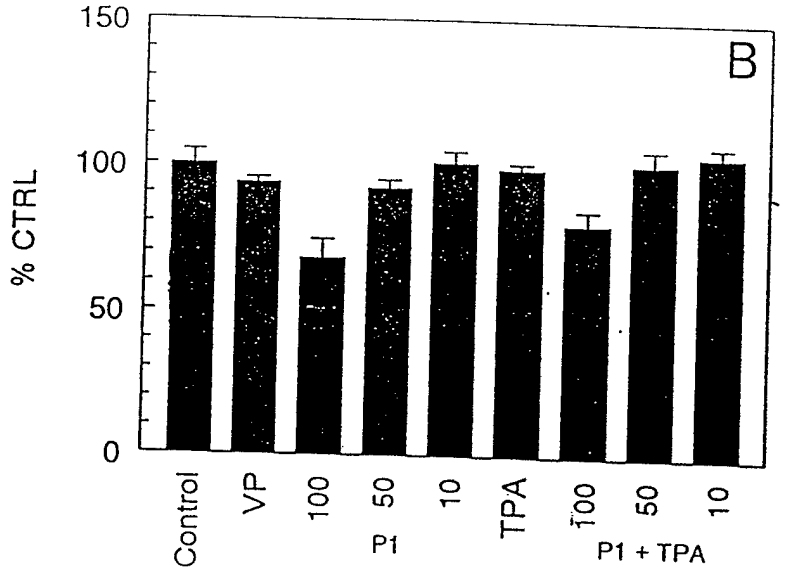
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FIGURE 6

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GROWTH AND TARGET RECOGNITION OF TUMOR INFILTRATING LYMPHOCYTES FROM HUMAN BREAST CANCER.

Murray JL*, Hudson M, Anderson BW, Castillega A, Singletary E, Kudelka A, Hortobagyi G, and Ioannides C, M.D. Anderson Cancer Center, Houston, Texas 77030.

Tumor infiltrating lymphocytes (TIL) and tumor associated lymphocytes (TAL) from solid tumors and malignant effusions respectively, were isolated from 29 breast cancer patients. Significant in vitro proliferation and expansion was observed in 22 of 29 distinct samples. TIL cultures were initiated using OKT3 mAb in the presence of moderate concentrations (25-50 U/ml) of IL-2 followed by 100 U/ml of TNF-α. TAL were not stimulated with OKT3 mAb. Six of six distinct TAL grew in culture as predominately $CD4^+$ lines. Fourteen of 21 (66%) of primary breast TIL expanded in culture, and were predominately of $CD8^+$ phenotype. Autologous tumor lysis was observed in seven of eight cases tested. Three of the eight TIL/TAL preferentially lysed autologous tumor. Four breast CTL lines lysed breast tumor line SKBR3-A2 supporting the recognition of peptides presented by HLA-A2. In addition to the HER-2 peptide GP2, peptides E75, C85, E89 and E90 were recognized indicating that multiple HER-2 epitopes are presented to breast TIL/TAL in the tumor environment. This may be of interest in developing vaccine strategies for therapeutic management of breast cancer. Supported by DAMD grant 17-94-J-4313 and the Nellie Connally Breast Cancer Research Fund.

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VACCINE IMPLICATIONS OF A NEW CYTOTOXIC T CELL (CTL)-RECOGNIZED ANTIGEN IN EPITHELIAL CANCERS.

Only HER2/neu, over-expressed in 30% of breast and ovarian cancers, has been shown to be a source of CTL-recognized peptides in epithelial cancers. We have recently found that tumor-associated lymphocytes (TAL) from these cancers also recognize peptides derived from the folate binding protein (FBP), over-expressed widely in epithelial tumors. To determine the most promising peptide vaccine strategy, immunodominant peptides from both antigens were compared. **Methods:** TAL were isolated from the malignant ascites or pleaural effusions of 6 consecutive HLA-A2+ ovarian (4) and breast (2) cancer patients and incubated in 50-100 IU/ml of IL-2. Initial 4-hr chromium-release assays were performed within 1 week. T2 cells were utilized to reconstitute T cell epitotes in 50 μg/ml of peptide. The HER2/neu peptides, E75 and GP2, and the FBP peptide, E39, were used with a negative peptide (NP) control. **Results:** Eleven assays with the 6 TAL cultures were performed at an effector:target ratio of 10-20:1. Specific lysis of NP-pulsed T2 was 1.9±2.6%, E39-10.1±3.6 %, E75-11.2±4.9%, and GP2-7.2±4.1%. Recognition of E39, E75, and GP2 was significant vs NP ($p<0.05$). E39 was lysed by all 6 TAL cultures while the HER2/neu peptides were variably recognized. E39 and E75 both promoted optimal lysis of T2 at 25-50 μg/ml. Both peptides were effective (>60%) at blocking the cytotoxicity of 3 TAL cultures for ovarian and breast tumor cells in cold target assays. CTL induction in 4 TAL lines with a single E39 or E75-stimulation revealed no significant difference in proliferation index or the increase in cytotoxicity at 38% and30%, respectively. **Conclusions:** Both HER2/neu and FBP are CTL-recognized antigen systems in ovarian and breast cancer. Immunodominant peptides from each are similar in recognition by T cells, effective dose range, and CTL induction. Since FBP over-expression is more prevalent, E39 should prove to be a superior single peptide vaccine. Additionally, a polyvalent peptide vaccine for epithelial cancers could be formed using dominant and subdominant peptides from both proteins.

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59th Annual Meeting
Milwaukee, Wisconsin
February 12-14, 1998

Submitted to Society of Surgical Oncology
9/3/97

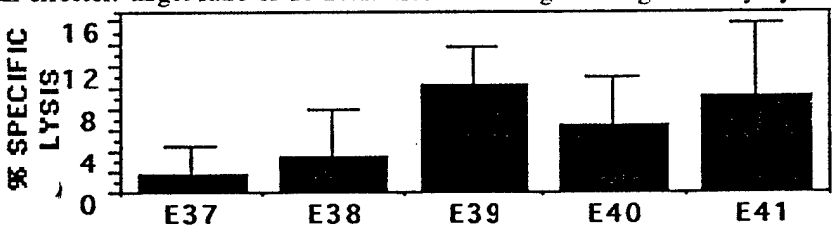
BREAST AND OVARIAN CANCER-ASSOCIATED LYMPHOCYTES RECOGNIZE FOLATE BINDING PROTEIN (FBP)-DERIVED PEPTIDES: VACCINE IMPLICATIONS.

G. E. Peoples*, B. W. Anderson, A. Kudelka, L. Murray, and C. G. Ioannides.
U. T. M. D. Anderson Cancer Center, Houston, TX.

BACKGROUND: Tumor-associated lymphocytes (TAL) isolated from breast and ovarian cancer patients contain cytotoxic T lymphocytes (CTL) capable of recognizing specific HLA/peptide complexes on tumor cells leading to tumor cell lysis. Currently, HER2/neu, over-expressed in only 30% of breast and ovarian cancers, is the only known source of CTL-recognized peptides in epithelial cancers. Therefore, we have investigated peptides derived from FBP which is over-expressed in >90% of ovarian cancers and the majority of epithelial tumors.

METHODS: TAL were isolated from the malignant ascites or pleaural effusions of 6 consecutive HLA-A2+ ovarian (4) and breast (2) cancer patients and incubated in 50-100 IU/ml of IL-2. Initial 4-hr chromium-release assays were performed within 1 week. T2 cells, incubated in 50 μg/ml of peptide, were used to reconstitute T cell epitopes. The FBP sequence was interrogated for HLA-A2 binding peptides, and 5 were synthesized.

RESULTS: Eleven assays with the 6 fresh TAL cultures were performed at an effector: target ratio of 10-20:1. E39 was recognized significantly by all



6 TAL cultures vs E37, E38, or unloaded T2 ($p<0.05$). E41 was strongly recognized but by only 3/6 TAL populations; therefore, we have focused on E39. Optimal T2 lysis was produced by E39 at 25-50 μg/ml. A single stimulation of 3 fresh TAL cultures with E39-pulsed T2 produced a 37±24% increase in proliferation and a 59±42% increase in cytotoxicity compared to unstimulated matched parallel TAL. TAL-mediated ovarian and breast tumor cell lysis was >60% blocked by E39-pulsed T2 in cold target inhibition assays.

CONCLUSIONS: The FBP-derived E39 and E41 peptides are recognized by freshly isolated TAL from ovarian and breast cancer patients suggesting in vivo expression and sensitization. Since FBP is over-expressed 20-fold in most adenocarcinomas, E39 may be the best currently known peptide to utilize in a peptide-based vaccine for epithelial tumors.

Supported by Grant DAMD-17-94-J-4313